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STUDIES IN THE PHYSIOLOGY OF THE FUNGI
XI. Bacterial Inhibition by Metabolic Products

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Considerable work has been done on the early phases of growth of bacteria in liquid media. Rahn ('06), Coplans ('07), Penfold ('14), Chesney ('16), Salter ('19), and others have shown quite definitely the factors involved in the lag phase of growth preceding the phase of logarithmic increase. They have demonstrated that the lag can be eliminated if the transfers are made during the period of logarithmic increase, but that certain factors such as difference in temperature, composition of the medium, or the age of the culture will produce a latent period immediately following the transfer.

Data on the later growth periods of bacteria are less extensive. Based on the total number of viable bacteria in the culture, the growth curve can be traced roughly as follows: It rises abruptly at first, which is the phase of logarithmic increase, then ascends more gradually until the peak is reached, and finally descends until the culture is sterile. The influence of inhibitory factors is most clearly seen in the later periods, those following the phase of logarithmic increase, the study of which is of fundamental and practical importance both in killing pathogenic bacteria, that is, hastening the decline in the growth curve, and in prolonging the life of useful cultures, suspending this decline. In the work presented here, emphasis is placed on the later periods of growth and on the influence of the products of a growing culture on the path of the growth curve.

Literature

The literature on the subject of the inhibition of bacteria in culture by their own metabolic products is widely scattered, and the investigational work in this phase of growth studies is very meager. The entire subject is often dismissed with some such statement as "the organisms are finally killed by their own products."

From time to time different investigators have sought to determine if there is a special metabolic product, enzymatic in nature, which inhibits the growth of the organism producing it. One of the earliest publications on this subject appeared by Eijkmann ('04). He grew *Bacillus coli* in gelatin at 37° C., treated it in different ways, and then solidified and reinoculated the gelatin. He concluded that *Bacillus coli* in gelatin produced a diffusible, thermolabile substance which would not pass through a porcelain filter and which inhibited growth of *Bacillus coli* and other organisms, for treatment with ether, subjection to heat, or filtration through a porcelain filter removed some inhibiting substance and permitted a streak growth on the solidified gelatin.

The following year Conradi and Kurpjuweit ('05,'05[a]) extended the work of Eijkmann, finding the same action in bouillon. They called the substance "autotoxin" and applied the theory to the germicidal action found in feces. They reported that the "autotoxin" of *Bacillus coli* was killed by boiling but was virulent up to a dilution of 1:3200 in a 10-hour culture, and that the heated stool filtrate from a paratyphoid patient would support growth of the same *Bacillus paratyphosis* in a 1:50 dilution, but the unheated filtrate only in a 1:400 dilution. Rolly ('06), Passini ('06), and Manteufel ('07) disputed the findings of Eijkmann, also those of Conradi and Kurpjuweit, and held that the existence of inhibitory substances had not yet been proved. Rolly could not repeat the work of Conradi and Kurpjuweit with the same results but found that the filtered half of a 20-hour bouillon culture gave better growth than the cooked half. Manteufel claimed that the loss of necessary food material from the media explained some of the results attributed to "autotoxin." Kruse ('10) summarized these reports and

explained the death of organisms in culture as probably due rather to the exhaustion of the media and the accumulation of well-known metabolic products than to an "autotoxin." He suggested the possibility of the exhaustion of the media and the accumulation of products causing the death of a few of the weaker individuals, which become self-digested, thereby releasing previously formed "autotoxin." Acids and alkalis are reported by him as inhibitory agents, although bouillon in which pneumococci had grown would not support a second growth even on readjusting the reaction.

In connection with some work on the latent period of growth, Chesney ('16) found that pneumococci in plain broth showed marked inhibition 24 hours after inoculation, the number of bacteria decreasing rapidly to zero, but if after 96 hours a portion of the bouillon was filtered through a porcelain filter and reinoculated, no inhibition was evident, indicating that the inhibitory substance was killed or attenuated in 3 days at 37° C.

It is apparent from the literature cited above that results are conflicting concerning the production of an enzymatic "autotoxin," and while the reports favoring the existence of such a product are not conclusive, no other satisfactory explanation for the observed reactions has been demonstrated.

Recent literature has indirectly contributed considerable of value concerning the relationship of acid and alkali to growth and death of bacteria, through the more general use, since 1916, of the hydrogen ion concentration as an expression of acidity of media. Winslow and Lochridge ('06), working on *Bacillus coli* and *Bacillus typhosus*, stated that the toxic effect of inorganic acids, HCl and H_2SO_4, corresponded to their dissociation, but with organic acids, acetic and benzoic, the undissociated molecule was also important, for results did not correspond to the dissociation of the acids. Michaelis ('14) advanced the idea that organisms produce acid to a certain concentration, which he found to be P_H 5.0 with *Bacillus coli* in lactose bouillon, and that they automatically protect themselves against harmful amounts.

Since that time a great deal has been published on final or limiting hydrogen ion concentrations for different organisms,

but only a very little on the effect on growth of changes in hydrogen ion concentration during growth. Clark ('15) determined the final P_H of 16 cultures of *Bacillus coli* in 1 per cent dextrose medium as P_H 4.67–5.16, and Clark and Lubs ('15) in constructing their media for differentiating the members of the colon-aerogenes group showed that a reversion of reaction toward the alkaline may take place, depending on the dextrose, but they did not show the relationship between reversion of reaction and growth. Itano ('16) reported that with *Bacillus subtilis*, *Streptococcus erysipelatus*, and *Streptococcus lacticus* in plain broth, acid was formed in alkaline media and alkali in the acid media, thus bringing the P_H to a certain definite hydrogen ion concentration. Fred and Loomis ('17) showed a wide range of reaction for *Bacillus radicicola*, obtaining good growth between P_H 3.9 and 11.1. They also demonstrated that the hydrogen ion concentration approaches the neutral point during growth. Shohl and Janney ('17) found that P_H 4.6–5.0 was inhibitory for *Bacillus coli* in urine. Ayers, Johnson, and Davis ('18) added streptococci to the list of organisms whose final P_H was demonstrated. They separated the pathogenic from the non-pathogenic forms on the basis of limiting hydrogen ion concentration, the former reaching P_H 5.4–6.0 and the latter P_H 4.6–4.7.

The work of Ayers and Rupp ('18) on simultaneous acid and alkali fermentations showed some interesting P_H curves. They found in a .5 per cent dextrose medium that *Bacillus coli* produced acid to P_H 4.8 but that *Bacillus aerogenes* produced less initial acid and the reaction reverted to P_H 6.5. From quantitative determinations of dextrose and of formic, acetic, lactic, and succinic acids, they explained the reversion of *Bacillus aerogenes* as a fermentation of the organic acids, mostly formic and acetic, to carbonates. With the alkali-forming milk bacteria, they showed alkaline fermentation of citrate, acid fermentation of dextrose, and a practically neutral reaction from the simultaneous fermentation of the citrate and dextrose. Gillespie ('18) found *Actinomyces chromogenus* gave a poor growth at P_H 4.8–5.2 and decreased the hydrogen ion concentration of the media during growth. Wyeth ('18) showed with *Bacillus coli* in glucose bouillon that the final P_H varied with

the initial P_H; i. e., P_H 7.11 progressed to P_H 5.70 and P_H 4.96 went to P_H 4.68. He also showed a difference in critical P_H according to the acid used, whether hydrochloric, acetic, or lactic acid. Wyeth ('19) extended his previous work and found in 2 per cent peptone that an initial range of P_H 4.29–9.37 gave a final range after 216 hours of P_H 5.92–8.55, and that with an initial P_H above 8.48 the production of acid exceeded that of alkali and the reaction approached P_H 8.48. Indole formation was completely inhibited by dextrose, partially by sucrose, and not at all by starch. Avery and Cullen ('19) used the final hydrogen ion concentration to separate strains of *Streptococcus hemolyticus;* 124 human strains attained a final P_H of 4.8–5.3, while 40 dairy strains reached P_H 4.3–4.5.

Considerable work has recently appeared on the pneumococcus. Cullen and Chesney ('18) showed the relation of the growth of pneumococcus in plain broth to hydrogen ion concentration. The bacteria increased to 420,000,000 per cc. in 13.8 hours and then decreased to 160 per cc. in 96 hours. The hydrogen ion concentration increased from P_H 7.70 to P_H 7.03,[1] but these investigators expressed the opinion that the increase in hydrogen ion concentration is not the sole cause of the cessation of growth. Avery and Cullen ('19[a]) showed some interesting reactions of pneumococcus to carbohydrates. One per cent of maltose, saccharose, lactose, galactose, raffinose, dextrose, or inulin produced a final P_H of about 5.0. With .4 per cent dextrose, as high an hydrogen ion concentration was attained in 48 hours as with 1 or 2 per cent dextrose. Pneumococcus differed from *Bacillus coli* in that it produced acid in plain broth, and growth ceased at about P_H 7.0. When this culture was readjusted to P_H 7.8 and reinoculated, no growth occurred unless carbohydrate was added, yet the filtrate from a dextrose culture at P_H 5.2 if readjusted to P_H 5.8, 7.0, or 8.0 would again return to P_H 5.2. Growth could only be initiated within certain limits, in carbohydrate media P_H 8.3–6.8 and in plain broth P_H 8.1–7.0. They concluded that the exhaustion of fermentable carbohydrate is only one of the many

[1] To avoid confusion, attention is called to the distinction between the concentration of the hydrogen ions and the symbolic P_H. A numerical increase in hydrogen ions is expressed as a decrease in terms of P_H.

factors involved in the complex phenomenon of growth inhibition.

Lord and Nye ('19) have demonstrated the relation of time to inhibitory action of hydrogen ion concentration with pneumococcus. They found that Pneumococcus Type I was killed in 1 hour at P_H 4.5–4.7, in 3 hours at P_H 5.3, and in 6 hours at P_H 6.15, but survived 6 hours at P_H 6.35, and that between P_H 6.8 and P_H 5.1 there was a direct relation between the P_H and the time required for the death of the pneumococcus. In mixtures of equal quantities of emulsions of washed pneumococci and buffer solutions of different hydrogen ion concentrations they observed very little dissolution of the bacterial cells between P_H 8.0 and P_H 7.0 or between P_H 5.0 and P_H 4.0, but noticed almost complete dissolution in the zone of P_H 6.5– 5.5.

Bunker ('19) published the results of investigations of *Bacillus diphtheriae* extending over several years. The hydrogen ion curves in sugar-free and in 1 per cent dextrose media agree very closely with those of *Bacillus coli* in the experimental work of this report. He also showed that toxin was only produced within a rather narrow hydrogen ion range, P_H 7.8–8.25. The best growth, measured by pellicle formation, was obtained when the initial reaction was P_H 7.3–7.5. Cohen and Clark ('19) investigated the effect of hydrogen ion concentration on the rate of growth of different organisms during the early part of the growth curve, the period of logarithmic increase. Cultures were inoculated into media adjusted over a wide range of varying initial hydrogen ion concentrations, and observed for the first 10 hours of growth. In general, the different organisms reacted similarly. The most marked effect of the hydrogen ion concentration on early growth was found near the critical acid and alkali zones. They reported that with *Bacillus coli* fermentative activity was checked in 1 per cent dextrose bouillon at P_H 5.0, but that growth in plain bouillon was checked at P_H 5.7. They noted evidence of inhibition which obscured their results, but they did not study the inhibitory factors; however, it was found that the period of lag was more pronounced in alkaline than in acid media.

Recent contributions from Besson, Ranque, and Senez ('19), while they do not involve hydrogen ion concentration, advance

some new ideas on sugar relations and fermentation. They worked with *Bacillus coli* in bouillon containing varying amounts of dextrose. With less than .4 per cent dextrose the sugar was all removed in 24 hours and the cultures were viable after 10 days, while with .4 per cent or over the cultures were sterile in 6 days. They reported that fermentation with gas commenced at the time multiplication of the organisms ceased, that acid production started at the same time, and that more than one-half the total acid is produced in 1 hour.

From the literature reviewed it would appear that a correlation of growth curves and P_H curves, with frequent observations during growth, rather than a study of final hydrogen ion concentration, would add to our knowledge of metabolic changes in hydrogen ion concentration and of inhibition during growth.

Technique

The experimental work was planned on the basis of a correlation of the growth of the bacteria with the changes in the hydrogen ion concentration of the media produced during growth. The technique was uniform throughout to make all results comparable. Cultures were grown in Florence flasks of 500, 1000, and 2000 cc. capacity, filled to one-half their capacities for the initial volume of media to insure a uniform and maximum surface. The basic bouillon for all the cultures, designated plain bouillon through the text, consisted of 2.5 per cent bacto-beef and 1 per cent bacto-peptone made up with distilled water according to the Digestive Ferments Company circular of December, 1916. This plain bouillon forms the basis for the different dextrose media, with a few exceptions which are noted in the data.

A culture of *Bacillus coli*, culture FG, kindly furnished from the Dairy Division, United States Department of Agriculture, was used throughout the experimental work with one exception, in which *Bacillus aerogenes*, culture VE from the same laboratory, was substituted.

To avoid the lag phase, transfers from stock agar were grown through two successive cultures of plain bouillon, and the inoculation was made from the second culture between 6 and 10 hours, during its period of logarithmic increase. A uniform tempera-

TABLE I

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI IN PLAIN AND 1 PER CENT DEXTROSE BOUILLON AT 30° C.

Hours	1 Plain bouillon		2 Plain bouillon and 1% dextrose	
	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	54,000	7.1	55,000	7.1
12	175,000,000	6.8	268,000,000	5.3
20			281,000,000	5.1
24	320,000,000	7.2	214,000,000	4.9
36	538,000,000	7.5	220,000,000	4.8
48	609,000,000	7.6	189,000,000	4.8
72	450,000,000	7.7	119,000,000	4.8
96	459,000,000	7.8	14,500,000	4.9
120	293,000,000	7.9	11,100	4.9
144	250,000,000	8.1	0	4.9
168	151,000,000	8.1		
192	156,000,000	8.3		
234	125,000,000	8.2		
276	115,000,000	8.3		
348	89,000,000	8.3		
492	69,000,000	8.3		
612	71,000,000	8.3		
1284	53,000,000	8.5		
1800	7,500,000	8.7		

ture of 30° C. was maintained for all cultures throughout the work.

The changes in growth and hydrogen ion concentration in each culture were followed by removing, under aseptic conditions, a 3-cc. sample at 12- or 24-hour intervals, after the flasks had been rotated briskly 30 times to mix the contents thoroughly. One cc. was diluted and plated in triplicate in agar composed of plain bouillon to which 1 per cent dextrose and 1.5 per cent granular agar had been added. The hydrogen ion concentration was determined from the remaining 2 cc. according to the colorimetric method of Clark and Lubs ('17), using the micro-colorimeter described by Duggar ('19). The hydrogen ion concentration is expressed in P_H, or the reciprocal values of Sörensen now in general biological use. Plates were counted after an incubation of 72 hours at 30° C.

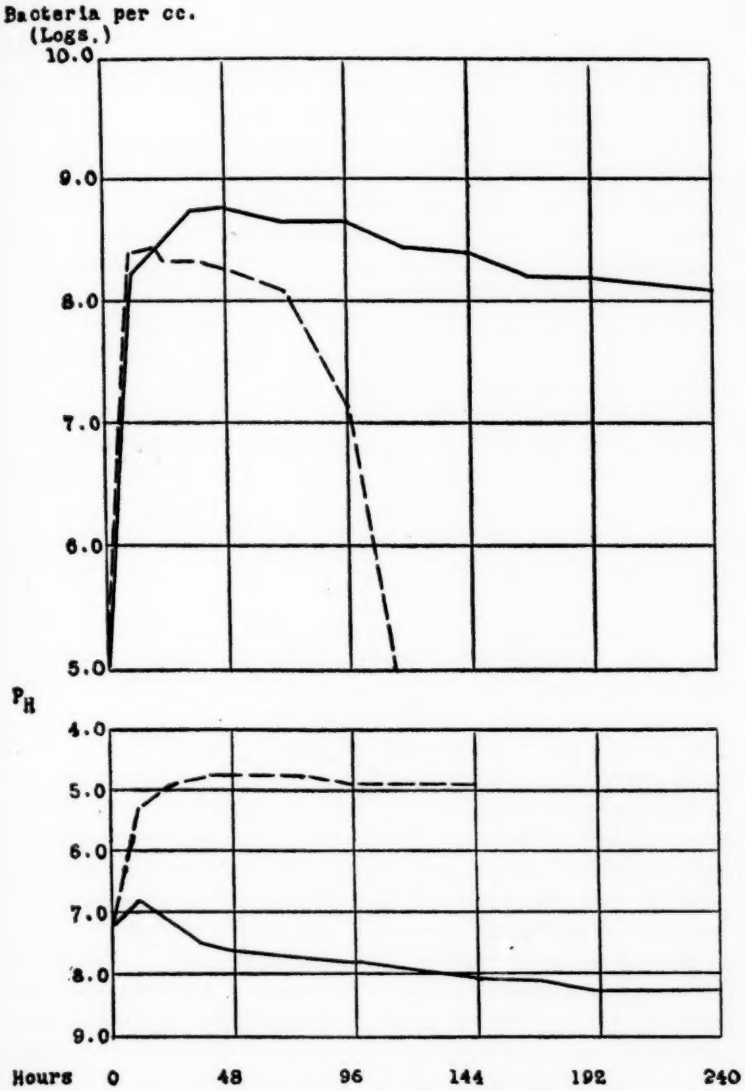

Fig. 1. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C.
———— plain bouillon.
— — — — 1 per cent dextrose bouillon.

EXPERIMENTAL DATA

As a starting point for the experimental work and as a basis for comparison of inhibitory action, one culture in plain bouillon and one culture in this bouillon with 1 per cent dextrose added were inoculated with equal numbers of *Bacillus coli* from the same culture. The resulting growth (expressed in numbers of bacteria per cc.), and the hydrogen ion concentration of the media (expressed in P_H) are recorded in table I. The comparison is more strikingly shown in fig. 1, in which the growth curves are plotted from the logarithms of numbers of bacteria per cc. as given in table I. A comparison of the hydrogen ion curves shows a rapid production of acid from dextrose, attaining P_H 4.8 in 36 hours, but a slower production of alkali in the plain bouillon with the exception of the short acid break at the beginning of the curve. Growth in the dextrose bouillon is more rapid in 12 hours than in the plain bouillon but the maximum is reached in 20 hours, 281,000,000 bacteria per cc. when the P_H is 5.1, and the decline is then very abrupt, terminating in sterility of the culture in 144 hours. In the plain bouillon, the maximum is reached in 48 hours, 609,000,000 bacteria per cc., with a P_H of 7.6. However, after 75 days, although a P_H of 8.7 is attained, there are still 7,500,000 viable bacteria per cc. in the culture. Apparently, then, the more intense inhibition is found in the dextrose rather than in the plain bouillon.

If a bacterial "autotoxin," or any inhibitory action such as Chesney found with pneumococcus in plain broth, is produced by *Bacillus coli*, it would seem, from the results given in table I, to be associated with the dextrose bouillon and not with the plain bouillon. A series of cultures in a 1 per cent dextrose medium were observed for the purpose of determining any variation in inhibitory action during growth and death. The results are given in table II and illustrated in fig. 2. Flask 1, the parent culture, contained the same 1 per cent dextrose bouillon as Culture 2 of table I. Subcultures of 200 cc. each were removed from the parent cultures at the times indicated, commencing before the point of maximum growth was reached and covering a range well into the period of rapid death. The reaction of the subcultures was readjusted to approximately neutral with sterile N/1 NaOH to eliminate the acidity factor,

TABLE II

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI IN 1 PER CENT DEXTROSE BOUILLON AT 30° C., SUBCULTURED AT INTERVALS

Hours	Parent culture		Subcultures									
			1		2		3		4		5	
	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	55,000	7.1										
12	268,000,000	5.3										
14			268,000,000	7.6								
20	281,000,000	5.1			281,000,000	7.1						
24	214,000,000	4.9	511,000,000	5.0	295,000,000	5.4						
26							214,000,000	7.5				
36	220,000,000	4.8	557,000,000	4.9	428,000,000	4.9	339,000,000	5.1				
48	189,000,000	4.8	488,000,000	4.9	396,000,000	4.8	342,000,000	4.8				
72	119,000,000	4.8	94,000,000	4.8	174,000,000	4.8	136,000,000	4.8	123,000,000	7.3		
84									261,000,000	6.7		
96	14,500,000	4.9	8,900,000	4.9	5,500,000	4.9	23,000,000	5.0	264,000,000	5.3	16,900,000	7.3
108											274,000,000	5.7
120	11,100	4.9	1,230,000	4.9	610,000	4.9	1,980,000	4.9	194,000,000	4.9	227,000,000	5.1
144	0	4.9	38,300	4.9	29,600	4.9	330,000	4.9	148,000,000	4.9	134,000,000	4.9
168			9,400	4.9	1,850	4.9	40,000	5.1	84,000,000	4.9	114,000,000	4.9
192			333	4.9	39	4.9	2,900	4.9	62,000,000	4.9	89,000,000	4.9
216			21	4.9	3	4.9	408	4.9	19,000,000	4.9	38,000,000	4.9
240			1	4.9	0	4.9	48	4.9	2,760,000	4.9		
264									350,000	5.1	70,000	4.9
288							0	4.9	14,000	5.0		
312									552	5.0	26,400	4.9
336									84	4.9		
360									9	4.9	310	4.9
408									0	5.0	0	4.9

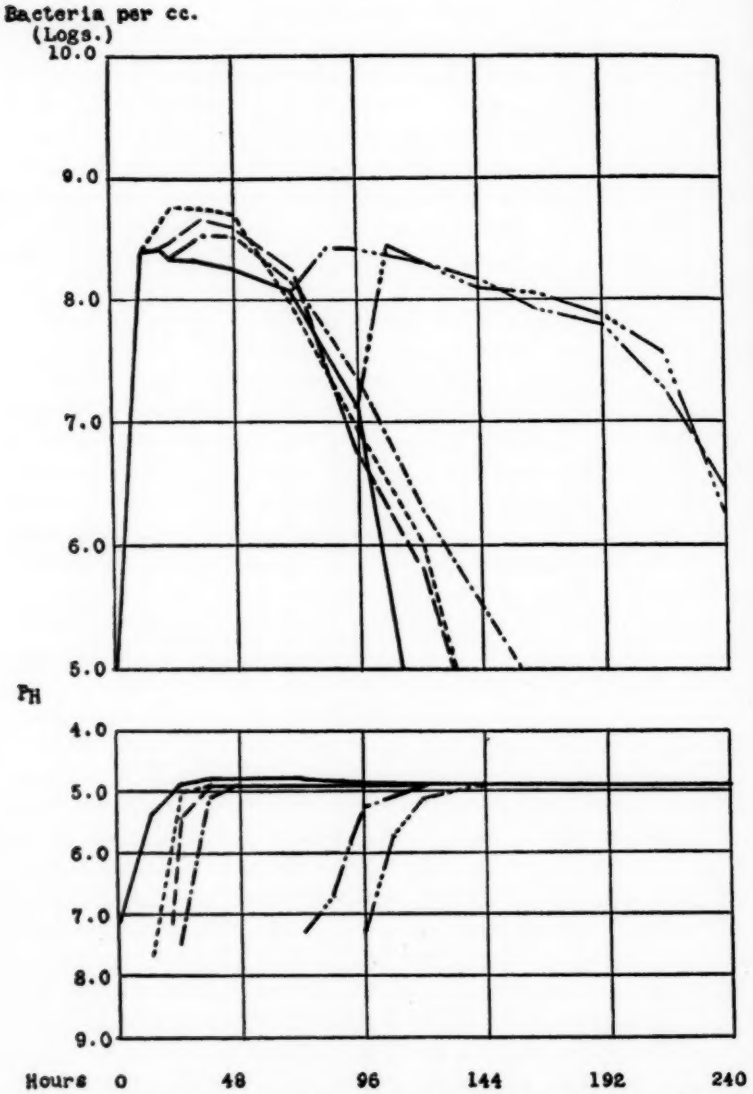

Fig. 2. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., 1 per cent dextrose bouillon, subcultured at intervals.

———— parent culture.
- - - - - - - - Subculture 1.
— — — — Subculture 2.
—.—.—. Subculture 3.
—..—..— Subculture 4.
—...—... Subculture 5.

and the subcultures were then observed as new cultures. The points of maximum growth for the different subcultures in order are 557,000,000, 428,000,000, 342,000,000, 264,000,000, and 274,000,000 bacteria per cc., so that the subcultures fall in a regular series of decreasing maximum growths, with the exception of Culture 4 whose maximum might have occurred unobserved between 84 and 96 hours. There is nothing distinctive in the changes in hydrogen ion concentration, each subculture producing acid to P_H 4.9 in 24 to 36 hours. It would seem, then, from this series of subcultures that some factor besides hydrogen ion concentration caused an inhibition of the growth, increasing with the age of the culture.

To determine the influence of the exhaustion of the medium as a factor in the inhibitory action shown in fig. 2, another similar series was observed. Four subcultures of 200 cc. each were removed from a parent culture at 96 hours and treated in different ways. All were readjusted to approximately neutral with N/1 NaOH. In addition 50 cc. of plain bouillon condensed 5 times was added to No. 2 (200 cc.), making a total volume of 250 cc. No. 3 received 50 cc. of the condensed bouillon and 1 per cent dextrose. No. 4 received the same nutrients as No. 3 and was then sterilized for 15 minutes at 120° C. to kill any "autotoxin" or inhibitory enzymatic substance, and reinoculated as a new culture. The results are contained in table III and fig. 3. The changes in hydrogen ion concentration are very uniform, falling on almost the same line, an increase until P_H 4.9 is reached in 48 hours. The exhaustion of the medium is shown, however, by the increased growth both in the culture with the bouillon replenished and in the replenished bouillon with dextrose added. The addition of dextrose shows almost no advantage over the addition of concentrated bouillon alone, so that dextrose is not considered an important factor at this time. Acid production from P_H 7.3 to 4.9 in Subcultures 1 and 2 where the dextrose was not replenished shows that all the dextrose had not been fermented in the parent culture at 96 hours. The maximum in Subculture 3 (table III) of 545,000,000 bacteria per cc. compares very favorably with 557,000,000 in Subculture 1 of table II, so that it would appear that the exhaustion of the nutrients contained in the plain bouillon was a very im-

TABLE III

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI IN 1 PER CENT DEXTROSE BOUILLON AT 30° C., SUBCULTURES WITH ADDED NUTRIENTS

Hours	Parent culture		Subcultures							
			1		2		3		4	
					Concentrated plain bouillon		Concentrated plain bouillon and 1% dextrose		Concentrated plain bouillon and 1% dextrose (sterilized and reinoculated)	
	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	82,000	6.8								
12	288,000,000	5.3								
24	250,000,000	4.8								
48	271,000,000	4.7								
72	87,000,000	4.7								
96	16,900,000	4.7	16,900,000	7.3	18,000,000	7.3	13,500,000	7.2	360,000	6.9
108			274,000,000	5.7	522,000,000	5.5	545,000,000	5.5	309,000,000	5.5
120	21,400	4.7	227,000,000	5.1	527,000,000	5.1	526,000,000	5.1	298,000,000	5.1
144	4,000	4.7	134,000,000	4.9	432,000,000	4.9	496,000,000	4.9	225,000,000	4.9
168			114,000,000	4.9	166,000,000	4.9	160,000,000	4.9	161,000,000	4.9
192			89,000,000	4.9	102,000,000	4.9	128,000,000	4.9	105,000,000	4.9
216			38,000,000	4.9	5,000,000	4.9	8,000,000	4.9	31,000,000	4.9
264			70,000	4.9	150,000	4.9	162,000	4.9	10,000	4.9
312			26,400	4.9	1,500	4.9	310	4.9	0	4.9
360			310	4.9	0	4.9	0	4.9		
408			0	4.9						

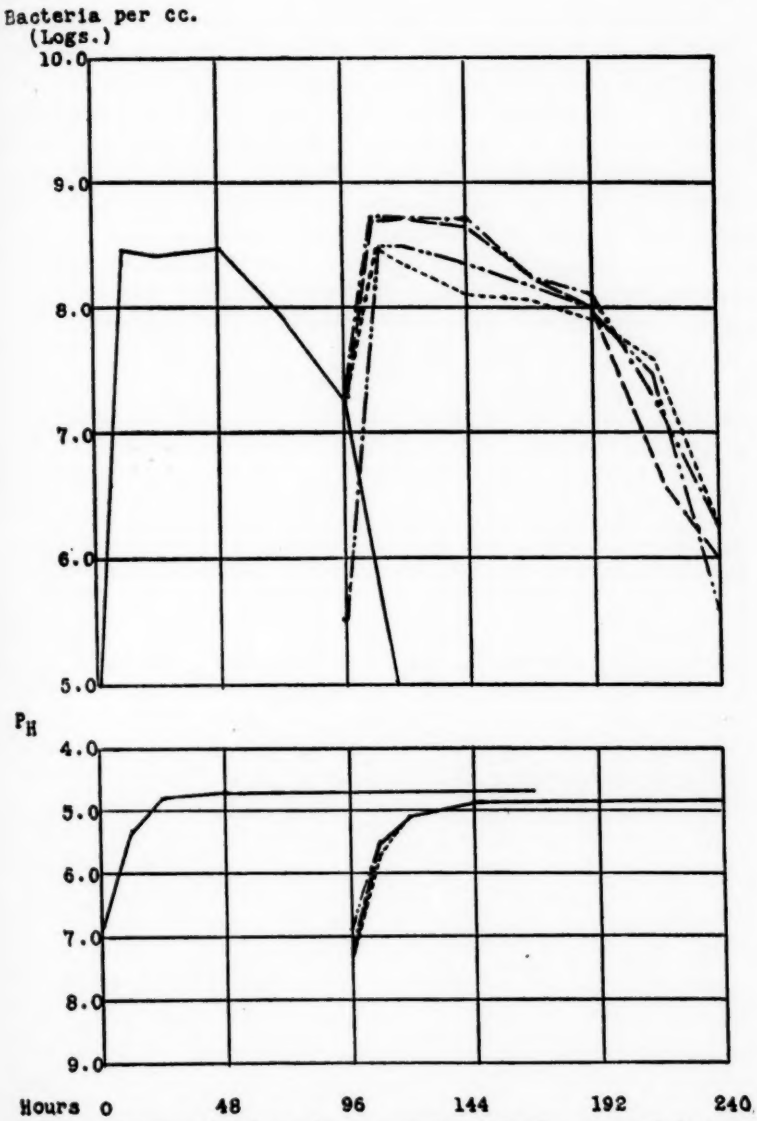

Fig. 3. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., 1 per cent dextrose bouillon, subcultures with added nutrients.

———— parent culture.
- - - - - - - - Subculture 1.
— — — — Subculture 2.
—·—·—· Subculture 3.
—·—·—·— Subculture 4.

portant factor in causing the increasing inhibitory action up to 96 hours. Subculture 4, with the same added nutrients as Subculture 3 but sterilized and reinoculated, did not attain the growth of Subculture 3, probably because of the small inoculation. However, the fact that the sterilized subculture did not surpass the unsterilized would indicate that in the parent culture or in the other subcultures the inhibition was not due to a substance which could be killed by sterilizing.

Some investigators have reported that the inhibitory action disappeared on standing and that a good growth was attained upon reinoculation, although the acidity was unaltered. To check this with *Bacillus coli*, 3 to 5 days after the cultures reported in table III became sterile, Subcultures 1, 3, and 4 were mixed together and divided into three equal 200-cc. portions, designated Cultures A, B, and C. Culture A was unchanged; Culture B was sterilized 15 minutes at 120° C.; and Culture C was readjusted to P_H 7.3. All were inoculated from the same culture of *Bacillus coli* with approximately 275,000 bacteria per cc. The growth and hydrogen ion concentration changes are recorded in table IV and fig. 4. Where unaltered, the hydrogen ion concentration in Cultures A and B is P_H 5.1 at inoculation, progressing to P_H 4.9 in a short time. Death of the bacteria occurs shortly, with very little difference between the sterilized and unsterilized cultures. In Culture C, unsterilized but with acidity corrected to P_H 7.3, growth and formation of acid occur similar to that in a new culture. Normal growth when the acidity was adjusted to neutral and no growth when it was not, both in the sterilized and unsterilized cultures, would indicate that no thermolabile substance which disappears on standing was present and that the hydrogen ion concentration of the medium was the important inhibitory factor.

The combined results expressed in the four tables might be summarized as follows: Inhibition to the point of death occurred only in dextrose bouillon in conjunction with acid formation, and not in plain bouillon with alkali formation. A slight inhibitory action was found in dextrose bouillon, increasing with the age of the culture up to 96 hours, which was not attributable to acid but which probably was due to a diminution of the nutrients in the medium. No indication was found of an in-

TABLE IV

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., DEXTROSE BOUILLON, REINOCULATED

Culture	A		B		C	
Treatment	None		Sterilized		Acidity adjusted	
Hours	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	275,000	5.1	286,000	5.1	261,000	7.3
12	11,400	5.0	15,200	4.9	280,000,000	5.5
24	7,200	5.0	3,800	4.9	320,000,000	5.5
36	3,600	4.9	650	4.9	340,000,000	5.3
48	530	4.9	75	4.9		
60	30	4.9	2	4.9	264,000,000	5.1
84	0	4.9	0	4.9	104,000,000	5.1
108					89,000,000	5.1
132					57,000,000	5.1
156					9,500,000	5.1
180					660,000	5.1
204					36,600	5.1
228					5,400	5.1
252					280	5.1
300					0	5.1

hibitory substance which was destroyed by sterilization or inactivated on standing. The evidence of these results is against an "autotoxin" theory and points toward the hydrogen ion concentration as the predominating inhibitory factor in the experiments cited.

The balance of the experimental work concerns the relation of hydrogen ion concentration to inhibition. To counteract the influence of acid and alkali produced during growth, and thus to study their action by comparison, two cultures were observed in which the acid or alkali formed was neutralized at frequent intervals. The media used was the same as that reported in table 1, one culture of plain bouillon and the other of 1 per cent dextrose bouillon, 500 cc. each in 1000-cc. flasks. The acid produced in the dextrose culture was neutralized at 12-hour intervals by the addition of N/1 NaOH and the hy-

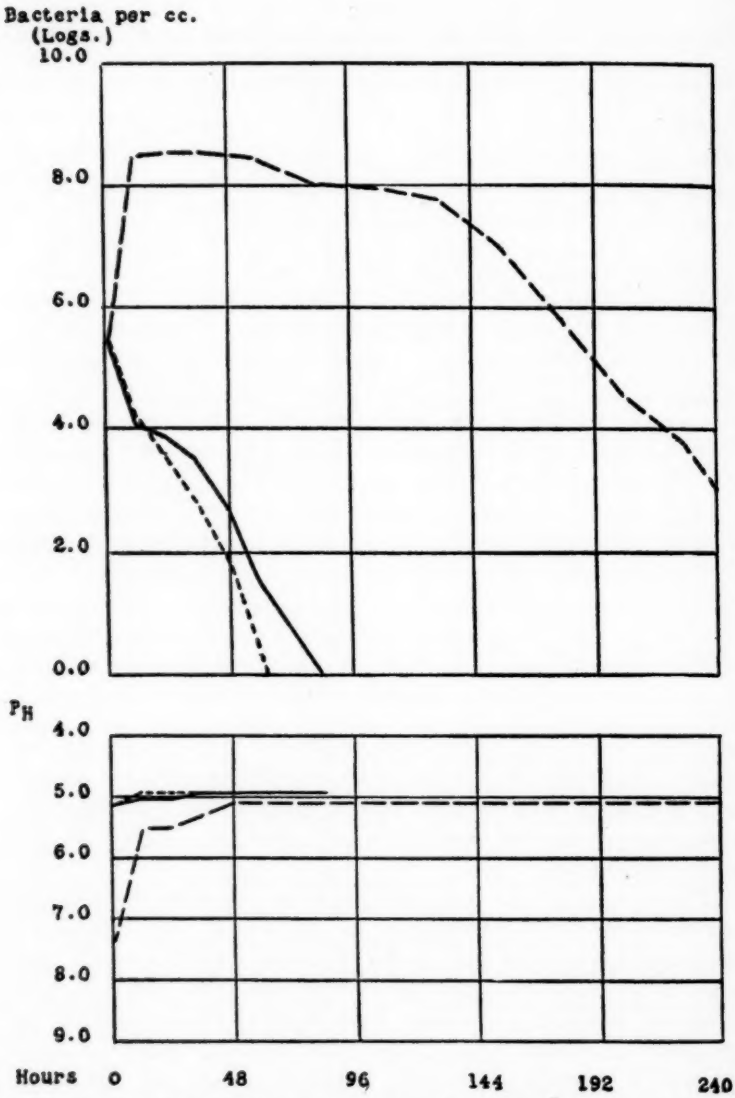

Fig. 4. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., dextrose bouillon, reinoculated.

———— Culture A, untreated.
- - - - - - - - Culture B, sterilized.
— — — — Culture C, acidity adjusted.

TABLE V

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., PLAIN AND 1 PER CENT DEXTROSE BOUILLON, NEUTRALIZED AT INTERVALS

Hours	1 Plain bouillon			2 1% Dextrose bouillon		
	Bacteria per cc.	Cc. of N/5 HCl added	P_H	Bacteria per cc.	Cc. of N/1 NaOH and N/5 HCl added	P_H
					N/1 NaOH	
0	57,000		7.1	54,000		7.1
12	184,000,000		7.0	259,000,000		5.3
14					4.0	6.5
24	391,000,000		7.3	347,000,000		5.1
26		3	6.9		6.3	7.1
36	552,000,000		7.4	507,000,000		5.1
38		5	7.0		6.5	6.9
48	660,000,000		7.3	612,000,000		5.1
50		5	6.7		7.0	7.0
60			7.1			5.3
62		3	6.7		7.0	6.7
72	631,000,000		7.1	692,000,000		5.7
74		3	6.7		7.0	7.3
96	684,000,000		7.2	728,000,000		7.5
					N/5 HCl	
98		3	6.8		3.0	7.3
120	696,000,000		7.3	532,000,000		7.3
122		3	6.7		3.0	7.3
144	680,000,000		7.3	455,000,000		7.5
146		3	6.9		16.0	6.9
168	570,000,000		7.2	487,000,000		7.5
192	693,000,000		7.1	618,000,000		7.3
234	437,000,000		7.3	885,000,000		7.5
236		3	6.9		15.0	7.0
276	450,000,000		7.1	794,000,000		7.6
278		4	6.8		20.0	7.1
348	327,000,000		7.1	608,000,000		7.7
350		3	6.5		25.0	6.7
492	256,000,000		7.1	441,000,000		8.1

drogen ion concentration was determined before and after each addition. The plain bouillon was treated similarly, correcting the alkali with N/5 HCl. The growth in bacteria per cc., the hydrogen ion concentration and the cc. of acid or alkali added

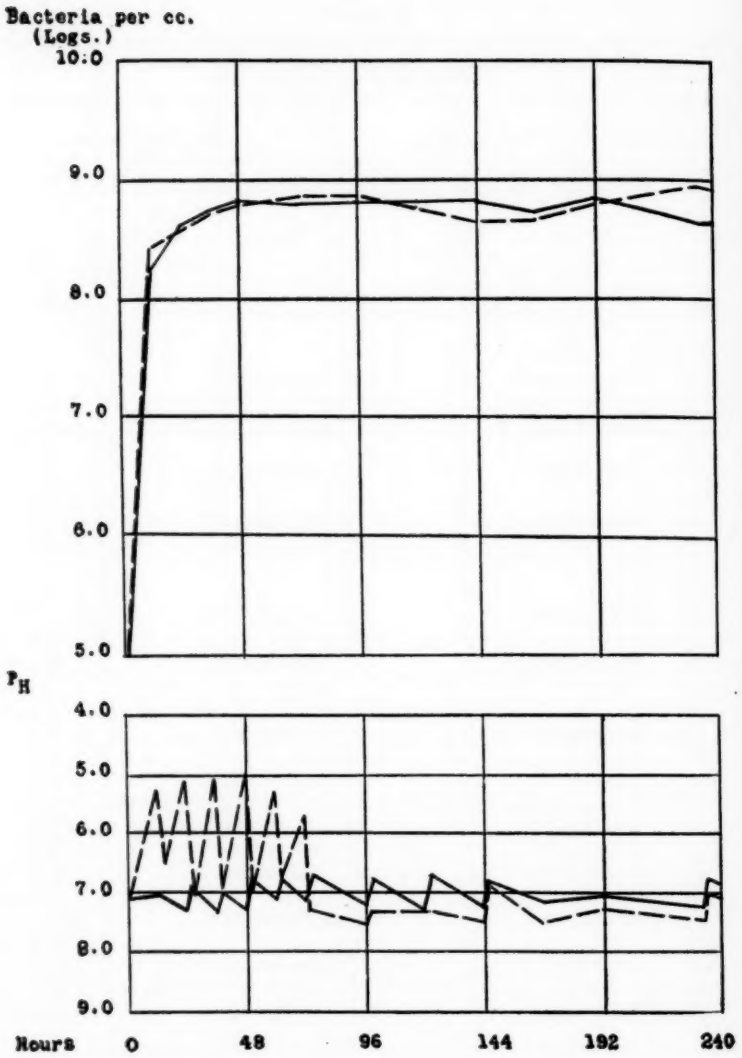

Fig. 5. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., plain and 1 per cent dextrose bouillon, neutralized at intervals.

———— plain bouillon.

— — — — 1 per cent dextrose bouillon.

are given in table v. A more striking representation of the changes in the concentration of the hydrogen ions is shown in the curves in fig. 5. Growth in both cultures is practically parallel, with the dextrose culture reaching the highest point—885,000,000 per cc. at 234 hours. From a comparison of these growth curves with those of fig. 1, it is quite evident that neutralizing the acid or alkali prolongs the growth at a higher level.

The P_H curve for the dextrose culture shows an abundant production of acid, going as high as P_H 5.1 several times. Between 72 and 96 hours, however, the formation changed to alkali, and N/5 HCl was added to neutralize. Table v shows that 37.8 cc. of N/1 NaOH were required to neutralize the acid from 1 per cent dextrose and that in the same time, 96 hours, 19 cc. of N/5 HCl were used in neutralizing the alkali in the plain bouillon, giving a ratio of 189 to 19, or approximately 10 to 1. Theoretically, then, one-tenth of the dextrose, or .1 per cent dextrose, would furnish just enough acid in 96 hours to neutralize the alkali formed in plain bouillon, and would hold at neutral the hydrogen ion concentration of a growing culture which was fermenting dextrose, if the dextrose were added in small amounts at frequent intervals.

On this basis a culture was started in plain bouillon. The amounts of dextrose added, the growth, and the P_H values are given in table vi and illustrated in fig. 6. By 72 hours the hydrogen ion concentration had demonstrated that the theoretical amount, .025 per cent of dextrose every 24 hours, did not furnish sufficient acid to neutralize the alkali, so the amount of dextrose was increased and the intervals between additions shortened to meet the needs of the culture. The reaction, with each addition of sugar, depends on the acid fermentation of the sugar and a subsequent alkali formation, as illustrated by the P_H curves between 48 and 72 hours and between 96 and 108 hours. This alkali formation was reversed by the addition of more sugar at the proper time. Although the theoretical calculation was upset by the increased growth, the P_H curve demonstrates that it was possible to hold the hydrogen ion concentration within a very narrow zone around the neutral point. The growth was very rapid, reaching 1,550,000,000 bacteria per cc. at 48 hours, or 2½ times as many bacteria as Culture 2,

TABLE VI

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., DEXTROSE ADDED AT INTERVALS

Hours	Bacteria per cc.	P_H	Cc. of 12.5% dextrose added	% Dextrose in 500 cc. of medium	Hours	Bacteria per cc.	P_H	Cc. of 12.5% dextrose added	% Dextrose in 500 cc. of medium
0	309,000	7.1	1.0	.025	252			1.2	.03
12	363,000,000	6.4			264	2,850,000,000	7.0	2.4	.06
24	923,000,000	7.0	1.0	.025	288		6.7	2.2	.055
36	1,360,000,000	6.9			312	1,700,000,000	6.7	2.0	.05
48	1,550,000,000	7.1	1.0	.025	336		6.6	1.0	.025
60	1,400,000,000	7.0			360		6.7		
72	1,512,000,000	7.3	1.0	.025	384	2,014,000,000	7.0	1.0	.025
84	1,230,000,000	7.3	1.5	.0375	408		7.0	1.0	.025
96	1,550,000,000	7.3	2.0	.05	432	1,840,000,000	7.1	1.0	.025
104		6.7			480		7.3	1.5	.0375
108		7.0	1.0	.025	504	1,705,000,000	7.2	1.8	.045
120	2,250,000,000	7.3	2.0	.05	532		7.2	1.5	.0375
132		6.8	1.0	.025	552	2,650,000,000	7.1	1.6	.04
144	3,125,000,000	7.0	1.5	.0375	564		6.5		
156	3,200,000,000	6.7	1.0	.025	576		7.0	1.6	.04
168	3,750,000,000	7.0	1.3	.0325	600	1,675,000,000	7.1	2.0	.05
180		6.9	1.2	.03	648		7.3	2.0	.05
192	3,250,000,000	6.9	1.2	.03	672	2,100,000,000	7.0	2.0	.05
204		6.9	1.1	.0275	720		7.2	2.0	.05
216	3,425,000,000	7.0	1.2	.03	744		6.7	1.0	.025
228		7.0	1.2	.03	768	1,500,000,000	6.7	1.0	.025
240	2,650,000,000	7.0	1.2	.03	816		7.1	1.3	.0325
					840	2,300,000,000	7.0	1.2	.03

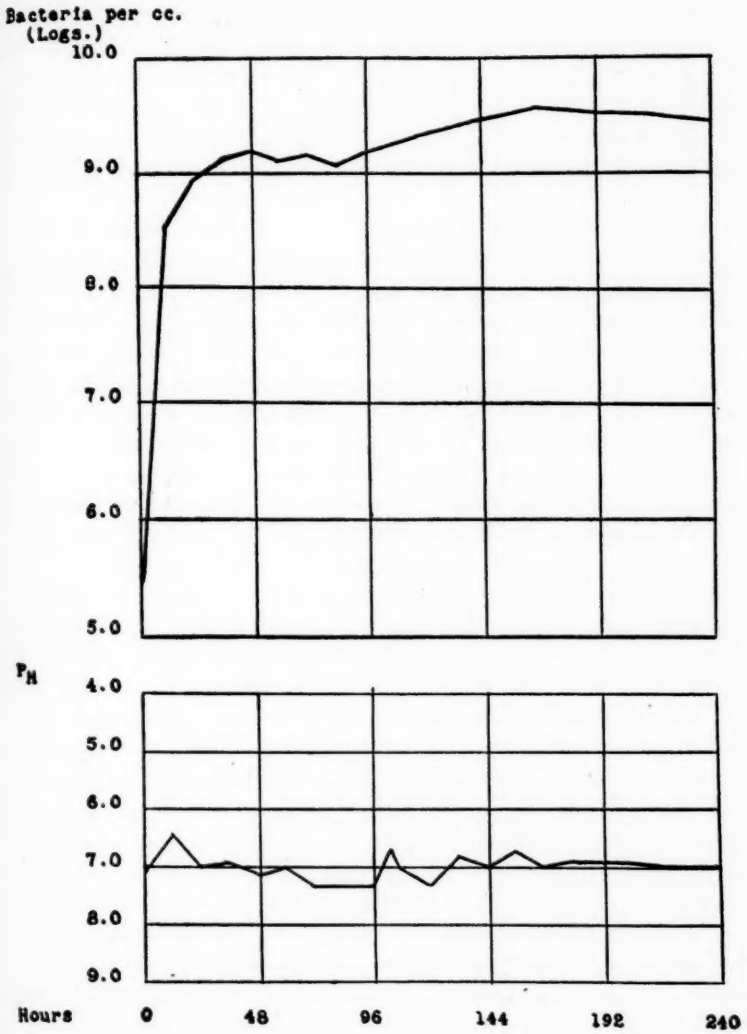

Fig. 6. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., dextrose added at intervals.

table v, produced in the same period of growth. Probably this increased growth explains the more rapid utilization of the dextrose than was calculated. The maximum growth was attained at 168 hours—3,750,000,000 bacteria per cc. Thus this culture showed the least inhibition of any of the experimental cultures and serves as a standard for comparison with the others.

To study more in detail the effect of small amounts of acid, a series of cultures was observed in which the only individual variation was in the initial amount of dextrose. To 250 cc. of plain bouillon in each of five 500-cc. flasks were added respectively .05, .1, .15, .2, and .3 per cent of dextrose, and all were inoculated from the same culture tube of *Bacillus coli*. The growth and changes in P_H are presented in table VII. The cultures are numbered, as indicated in the table, from 1 to 5 in order of increasing amounts of dextrose. Cultures 2, 4, and 5 are plotted in fig. 7 as representative of the series. The P_H curves show that acid was produced in each culture and that the amount of acid formed corresponded to the amount of dextrose provided. The cultures formed a regular series of increasing acidities. Following the acid production there was a reversion of the reaction toward alkalinity which was quite rapid in the first four cultures but slower in Culture 5, where a P_H of 5.1 was maintained from 24 to 96 hours. Comparing the growth curves of the five cultures during the period from inoculation to 48 hours, it is seen that Culture 2 makes the best growth and that Cultures 3, 4, and 5 follow in order. It would appear, then, that .1 per cent of dextrose or less is stimulative in effect and that there is no acid injury from a short exposure to P_H 5.9 (Culture 2). There is, however, some acid inhibition from a P_H of 5.5 (Cultures 3 and 4) and quite a marked inhibition—sufficient to cause some decrease in numbers—from 3 days' exposure to P_H 5.1 (Culture 5). In each case the growth curve ascended as the P_H curve descended toward the alkaline side. The maximum growth was approximately the same for all the cultures—1,400,000,000 to 1,800,000,000 bacteria per cc.—and was reached when the hydrogen ion concentration fell in a zone between P_H 7.0 and 7.6. At the point of maximum growth the hydrogen ion concentrations for the cultures in order were

TABLE VII

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., VARIATION IN INITIAL DEXTROSE

Cultures	1		2		3		4		5	
Dextrose	.05%		.10%		.15%		.20%		.30%	
Hours	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	300,000	7.1	340,000	7.1	325,000	7.1	335,000	7.1	270,000	7.1
12	392,000,000	6.4	399,000,000	5.9	321,000,000	5.5	329,000,000	5.5	333,000,000	5.4
24	992,000,000	6.9	1,060,000,000	6.7	881,000,000	6.1	593,000,000	5.5	529,000,000	5.1
36	1,392,000,000	7.3	1,768,000,000	7.1	1,200,000,000	6.8	1,132,000,000	6.0	624,000,000	5.1
48	1,376,000,000	7.5	1,330,000,000	7.4	1,292,000,000	7.3	1,496,000,000	7.1	648,000,000	5.1
72	1,360,000,000	7.6	1,360,000,000	7.6	1,680,000,000	7.6	1,824,000,000	7.5	512,000,000	5.1
96	853,000,000	8.1	960,000,000	8.2	1,270,000,000	8.2	1,350,000,000	8.2	454,000,000	5.1
120	516,000,000	8.4	520,000,000	8.5	672,000,000	8.5	472,000,000	8.4	516,000,000	5.2
144										5.5
168	295,000,000	8.5	275,000,000	8.5	243,000,000	8.5	248,000,000	8.5	635,000,000	5.7
192										5.9
216									1,055,000,000	6.9
240	166,000,000	8.5	169,000,000	8.5	160,000,000	8.5	224,000,000	8.5	1,655,000,000	7.4
288									948,000,000	8.1
336										8.5
360									252,000,000	8.5

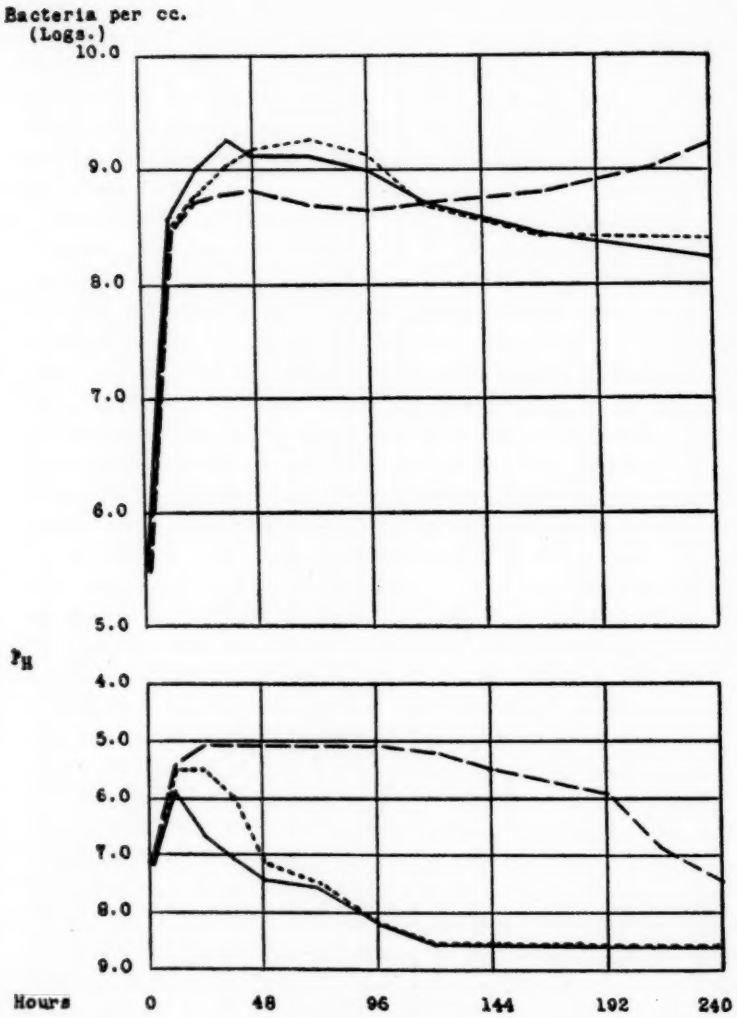

Fig. 7. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., variation in initial dextrose.

———— .1 per cent dextrose.
- - - - - - - - .2 per cent dextrose.
— — — — .3 per cent dextrose.

P_H 7.3, 7.1, 7.6, 7.5, and 7.4. The relation between growth curves and P_H curves, fig. 7, would indicate that *Bacillus coli* is more sensitive to alkali than to acid and that amounts of alkali or acid considerably less than the fatal dose become prominent factors in inhibiting growth.

Supplementing the preceding table, table VIII gives the results of growth of *Bacillus coli* in 1 per cent, 2.5 per cent, and 5 per cent dextrose media. A synthetic bouillon was used for these cultures consisting of .5 per cent asparagin, .5 per cent K_2HPO_4, and the dextrose as indicated. The growth and hydrogen ion concentration curves are plotted in fig. 8 on the same basis as the curves in all the other figures. As might be expected, the action in general corresponded to that of Culture 2, fig. 1, which was grown in 1 per cent dextrose. Both the growth and P_H curves showed a small lag at the beginning in 2.5 per cent dextrose and a greater one with some decrease in growth in 5 per cent dextrose. Following the initial lag, the cultures produced the usual growth, acid fermentation, and death. A slightly greater acid production occurred in the 5 per cent dextrose, for the hydrogen ion concentration went to P_H 4.7. The data of tables VII and VIII show that in cultures of *Bacillus coli* sufficient acid to kill the organisms was formed from 1 per cent or more of dextrose, while .15 to .3 per cent supplied only enough acid to inhibit the growth, and .1 per cent exerted a stimulative action. Thus the amount of dextrose present seems to regulate the reaction, which is a strong factor in growth and inhibition.

In connection with the reversion of reaction, the growth and inhibition of *Bacillus aerogenes* are of interest. One culture of plain bouillon and one culture of plain bouillon plus 1 per cent dextrose were inoculated with *Bacillus aerogenes;* the growth and hydrogen ion concentration changes are recorded in table IX and fig. 9. Both the growth and hydrogen ion concentration were very similar to those of *Bacillus coli* from the time of inoculation up to 96 hours. As fig. 9 illustrates, at 96 hours the abrupt descent of the growth curve was checked at 7,600,000 bacteria per cc. The slight drop to 5,000,000 bacteria per cc. in the next 48 hours was followed by a second rise which culminated in a maximum of 1,017,000,000 bacteria per cc. at 696 hours.

TABLE VIII

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., VARIATION IN INITIAL DEXTROSE IN ASPARAGIN BOUILLON

Cultures	1		2		3	
Dextrose	1.0%		2.5%		5.0%	
Hours	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	45,000	6.6	63,000	6.4	46,000	6.4
12	5,450,000	6.6	100,000	6.4	60,000	6.3
16	46,500,000	6.5	80,000	6.3	60,000	6.2
20	326,000,000	5.7	80,000	5.8	60,000	5.9
24	350,000,000	5.5	145,000	5.7	25,200	5.9
36	464,000,000	5.4	17,000,000	5.8	8,400	5.9
48	416,000,000	4.9	345,000,000	5.6	57,600	5.8
60	280,000,000	4.9	384,000,000	4.9	1,700,000	5.7
72	178,000,000	5.3	323,000,000	4.9	40,000,000	5.7
84	108,000,000	5.3	348,000,000	4.9	170,000,000	5.1
96	51,000,000	5.3	320,000,000	4.9	270,000,000	4.9
108	28,000,000	5.3	180,000,000	4.9	260,000,000	4.9
120	1,800,000	5.3	187,000,000	4.9	380,000,000	4.7
132	2,800,000	5.3	120,000,000	4.9	224,000,000	4.7
144	200,000		31,000,000		142,000,000	4.7
156	18,400	5.3	24,000,000	4.9	161,000,000	4.9
168	1,000	5.3	6,000,000	5.2	158,000,000	5.1
180	346	5.3	2,000,000	5.2	132,000,000	5.1
192	144	5.3	360,000	5.0	102,000,000	4.9
204	93		62,000		104,000,000	
216			391	5.2	54,000,000	5.3
240					21,000,000	5.3
264					9,900,000	5.0
300					900	5.0

Some points of interest in the P_H curves are that the high point, P_H 4.7, at 120 hours, did not occur during the period of greatest decrease in growth, that the P_H held at 4.9 for a considerable period after the second increase in growth began, and that the hydrogen ion concentration at the time when the maximum growth was reached was P_H 7.1. In plain bouillon there was no essential difference in growth or changes in hydrogen ion

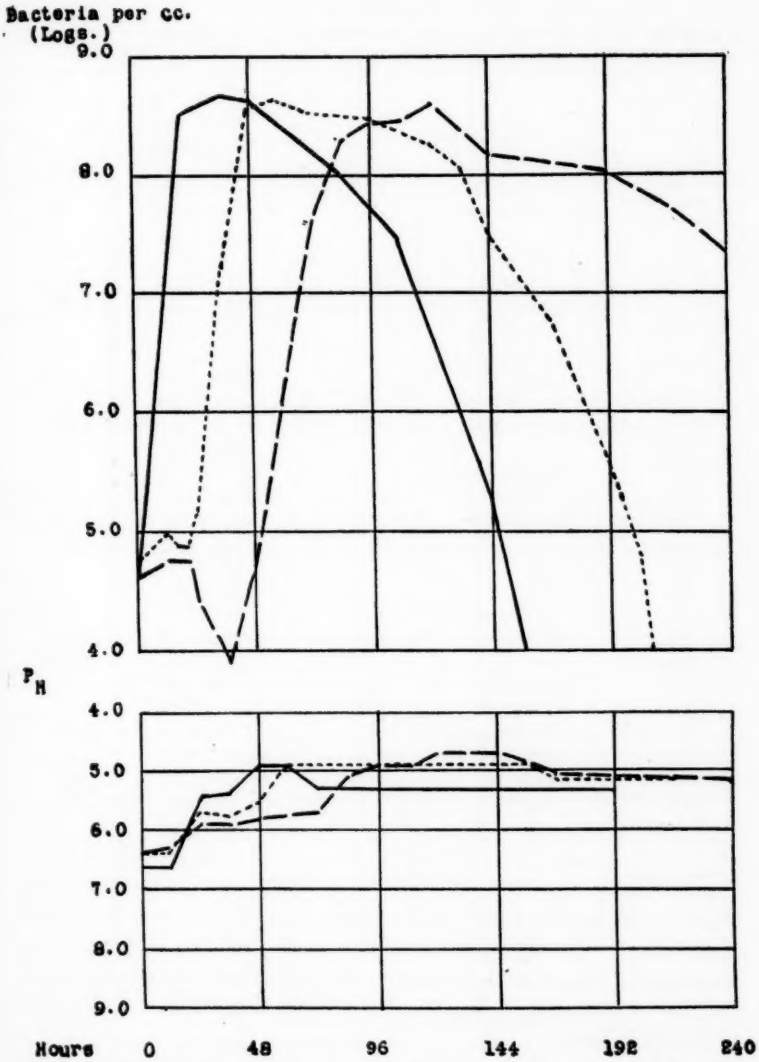

Fig. 8. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., variation in initial dextrose in asparagin bouillon.

——— 1.0 per cent dextrose.
- - - - - - - - 2.5 per cent dextrose.
— — — — 5.0 per cent dextrose.

TABLE IX

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS AEROGENES AT 30° C., PLAIN AND 1 PER CENT DEXTROSE BOUILLON

Hours	1 Plain bouillon		2 Plain bouillon and 1% dextrose	
	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	98,000	6.9	94,000	6.9
12	171,000,000	6.9	272,000,000	5.5
24	236,000,000	7.0	300,000,000	4.9
48	306,000,000	7.4	139,000,000	4.8
72	455,000,000	7.8	27,000,000	4.8
96	307,000,000	8.1	7,600,000	4.8
120	169,000,000	8.3	6,300,000	4.7
144		8.3	5,000,000	4.9
168	162,000,000	8.3	10,000,000	4.9
192	121,000,000	8.3	23,000,000	4.9
216	108,000,000	8.3	38,000,000	4.9
264			125,000,000	4.9
312			211,000,000	4.9
360			539,000,000	5.8
408	37,000,000	8.3	815,000,000	6.6
456	44,000,000	8.5	836,000,000	6.9
504			761,000,000	6.9
552	36,000,000	8.6	775,000,000	6.9
624	20,000,000	8.7	927,000,000	6.9
696	22,000,000	8.7	1,017,000,000	7.1
864	8,640,000	8.7	690,000,000	7.3
1032	2,520,000	8.7	643,000,000	7.0
1200	7,830,000	8.7	485,000,000	7.6

concentration between *Bacillus coli* (table I, Culture 1) and *Bacillus aerogenes* (table IX, Culture 1). There is a similarity in growth of *Bacillus coli* in .3 per cent dextrose and *Bacillus aerogenes* in 1 per cent dextrose, the difference between the organisms apparently being in the greater resistance of *Bacillus aerogenes* to acid.

While the experimental data reported above have emphasized the importance of the H and OH ions as factors in inhibition, these ions do not represent the only products of metabolism which might be considered as inhibitory to growth. Ayers and

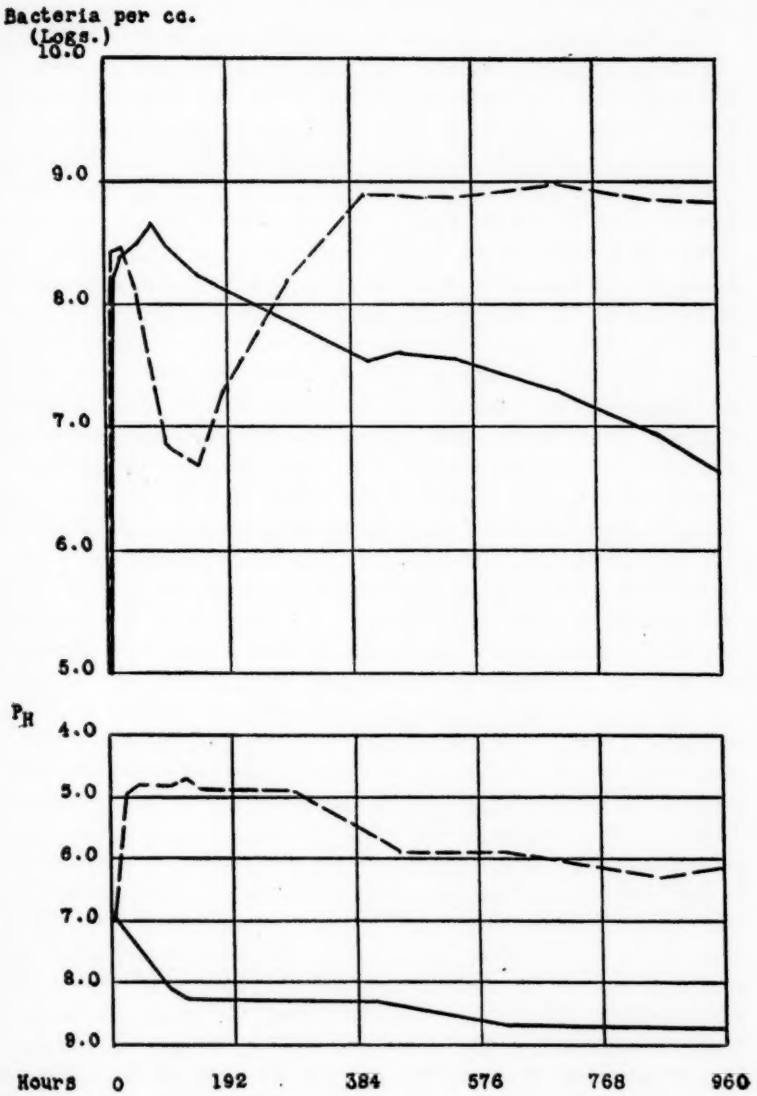

Fig. 9. Growth and hydrogen ion concentration of *Bacillus aerogenes* at 30° C.
———— plain bouillon.
— — — — 1 per cent dextrose bouillon.

Rupp have made quantitative determinations of formic, acetic, lactic, and succinic acids from *Bacillus coli* in a dextrose bouillon, and Wyeth, and Cohen and Clark have shown that the critical hydrogen ion concentration varies with the different acids, hydrochloric, acetic, and lactic, indicating that the anions of the acids or perhaps the undissociated molecules, as Winslow and Lochridge suggested, are also concerned in inhibition. Most of the work on the inhibitory effect of different acids has been based on inoculation of media of different P_H values obtained by using different acids, and the inhibition has been determined according to the presence or absence of growth after a certain interval. Such a method does not take into consideration milder phases of inhibition which are not severe enough to cause the death of the organisms. To illustrate this phase and to indicate some of the relations of the hydrogen ion factor to the other factors, the results of an experiment are presented in table x and fig. 10. Culture 1 was grown in 1 per cent dextrose bouillon, and Culture 2 in plain bouillon to which sterile N/5 HCl was added, as indicated in table x, in an attempt to simulate in plain bouillon the P_H curve of a culture fermenting dextrose bouillon, such as Culture 1. As seen from fig. 10, the culture produced alkali continually so that it was only possible by frequent additions of acid to hold the P_H in a zone around P_H 4.8, the greatest hydrogen ion concentration which Culture 1 attained. The growth curve, fig. 10, shows marked acid inhibition with almost no further increase in growth after the first addition of acid at 14 hours. There is practically no difference in the growth curves of the two cultures up to 72 hours, but from that point they separate widely, for death occurs shortly in the dextrose media and growth in Culture 2 does not go below 26,000,000 bacteria per cc. Thus a hydrogen ion concentration of P_H 4.8–4.9 when produced by the acid fermentation of dextrose was fatal, while that of P_H 4.7–5.1 from HCl was only strongly inhibitory, indicating that the other metabolic products of dextrose fermentation, such as acetate or lactate ions, evidently enter as factors in causing the death of the culture.

TABLE X

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., 1 PER CENT DEXTROSE BOUILLON AND PLAIN BOUILLON+HCl AT INTERVALS

Hours	1 1% dextrose bouillon		2 Plain bouillon+HCl		
	Bacteria per cc.	P_H	Bacteria per cc.	Cc. of N/5 HCl added	P_H
0	55,000	7.1	57,000		7.1
12	268,000,000	5.3	179,000,000		6.7
14				15	5.2
20	281,000,000	5.1			
24	214,000,000	4.9	178,000,000		5.3
26				4	5.0
36	220,000,000	4.8	197,000,000		5.2
38				5	4.9
48	189,000,000	4.8	175,000,000		5.1
50				5	4.7
60					4.9
72	119,000,000	4.8	116,000,000		5.0
74				3	4.7
96	14,500,000	4.9	77,000,000		4.9
120	11,100	4.9	96,000,000		5.3
122				3	4.9
144	0	4.9	55,000,000		5.1
146				2	4.9
168			26,000,000		4.9
192			60,000,000		5.1
194				2	4.9
234			74,000,000		4.9
276			144,000,000		5.3
278				4	4.9
348			148,000,000		5.1
350				3	4.8
492			95,000,000		4.9
612			73,000,000		5.1

Discussion

For a discussion of the combined results embodied in the experimental data presented, the inhibitory products of metabolism of *Bacillus coli* are divided under four topics: "autotoxins"; H ions; OH ions; and products of dextrose fermentation other than those directly related to changes in H ion concentration.

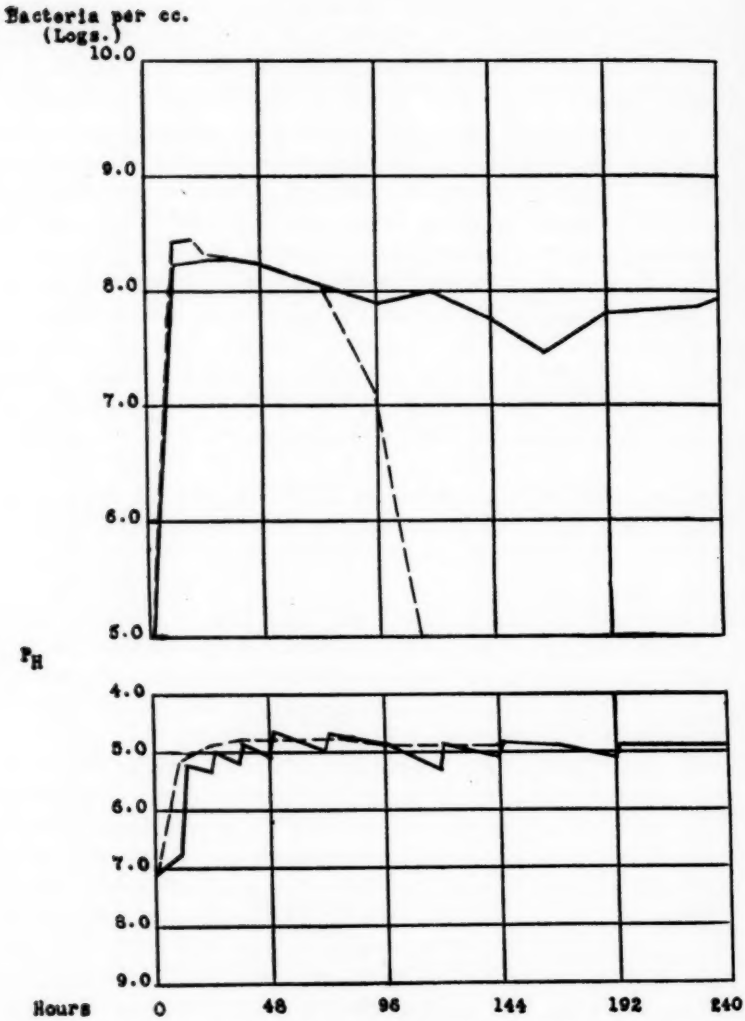

Fig. 10. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C.
———— plain bouillon+HCl at intervals.
— — — — 1 per cent dextrose bouillon.

It appears improbable that *Bacillus coli*, grown under the conditions of the experiments reported, produces any "autotoxin" or special inhibitory substance such as Eijkmann and others claimed. The results given in tables II and III indicate that the slight inhibition increasing with the age of the culture is probably associated with a diminution in nutrients. No thermolabile product could be detected which would inhibit growth if the hydrogen ion concentration were corrected. It is difficult to reconcile the production of an enzymatic inhibiting substance with such growth as appears in figs. 1 and 6, especially as death did not occur in these cultures. In plain bouillon, fig. 1, the culture was viable after 75 days; and in dextrose bouillon, fig. 6, with the hydrogen ion factor controlled, growth attained 3,750,000,000 bacteria per cc. in 7 days, and there were still present over 2,000,000,000 bacteria per cc. after 840 hours. In addition there was no indication in the cultures in which death occurred that death could be attributed to an "autotoxin."

There is a direct relation between hydrogen ion concentration and inhibition. If the acid is formed from the fermentation of dextrose, with *Bacillus coli*, fig. 7 and table VII, there is no indication of acid inhibition at P_H 5.9 if maintained for only a short time. Some inhibition is apparent at P_H 5.5 which increases with the time that the culture is exposed to this P_H. There is a marked inhibition from an exposure of 72 hours to P_H 5.1, fig. 7, but it is insufficient to cause death. Fig. 4, however, shows that a prolonged hydrogen ion concentration of P_H 5.1 is lethal, and in every case throughout the experimental work a P_H of 4.9, when produced by acid fermentation of dextrose, proves fatal, figs. 1, 2, 3, 4, and 8.

To illustrate the relationship between hydrogen ion concentration and growth, four curves from figs. 1, 6, and 7 are assembled in fig. 11. The highest growth curve, No. 2, is attained in the culture in which the P_H remains practically neutral; the P_H of 5.1, No. 3, produces an intermediate growth; and the slight difference in hydrogen ion concentration between P_H 5.1, No. 3, and 4.9, No. 4, is fatal.

The OH ions also prove to be inhibitory according to the plain bouillon growth curve in fig. 11. An alkalinity corre-

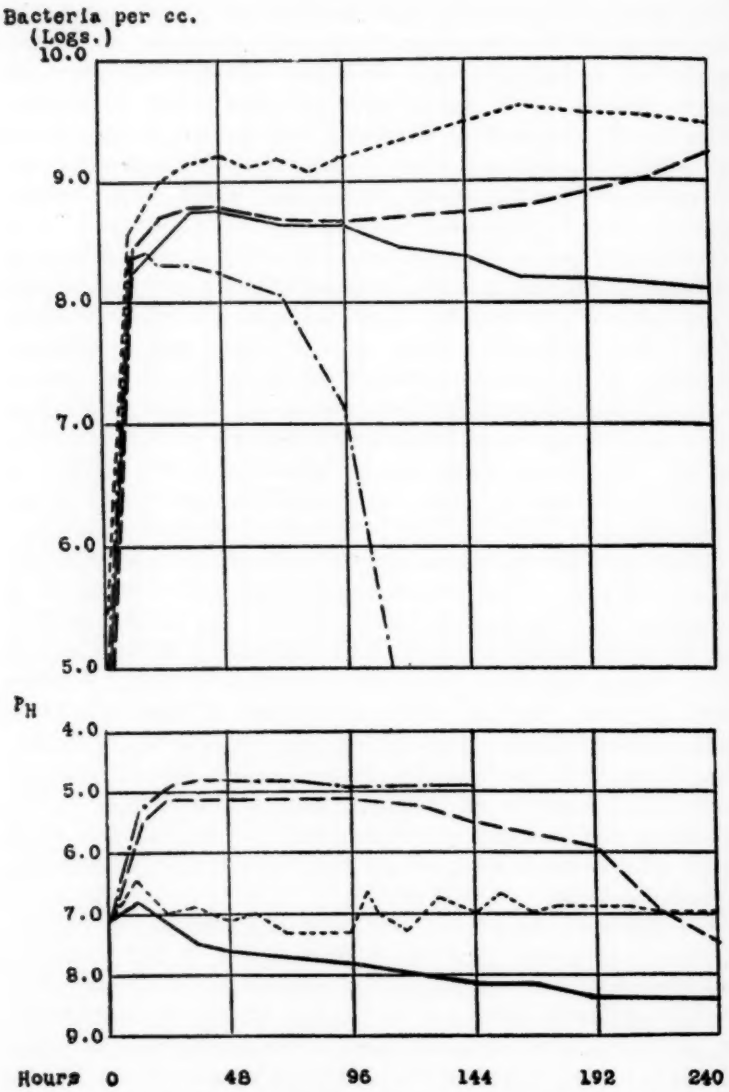

Fig. 11. Relation of growth to hydrogen ion concentration of *Bacillus coli* at 30° C.

No. 1 ———— plain bouillon.
No. 2 - - - - - - - - dextrose added at intervals.
No. 3 — — — — .3 per cent dextrose.
No. 4 —·—·—· 1 per cent dextrose.

sponding to P_H 7.6–7.8 is comparable in toxicity with an acidity of P_H 5.1. *Bacillus coli* seems more sensitive to small amounts of alkali than to small amounts of acid, for in the reversions of reaction in fig. 7, inhibition is evident shortly after crossing the neutral line, about P_H 7.1–7.6. In a freshly inoculated culture without dextrose, fig. 7, inhibition is first noted about P_H 7.5. While the hydroxyl ions appear more toxic to *Bacillus coli* in less concentration than the hydrogen ions, they do not seem to be fatal in greater concentration, for death of the culture was not observed on the alkaline side, although one culture containing $CaCO_3$, which is not reported in the data, carried the P_H to 9.5.

The importance of the factors other than H or OH ions which may enter into the inhibition or killing of a culture of *Bacillus coli* is not overlooked, but it should not be over-emphasized. For example, in a 1 per cent dextrose bouillon culture, such as is shown in fig. 1 or 10, in addition to the H ions, the anions, formate, acetate, lactate, and succinate, are formed (Ayers and Rupp, '18), probably other anions, and also the undissociated acids. These add their inhibitory action to that of the H ions in producing death at P_H 4.9, illustrated by fig. 10. The best growth curve, however, fig. 11, has only the hydrogen ion concentration controlled and in reality ferments much more dextrose than the 1 per cent dextrose culture of fig. 10. The former culture ferments a total of over 1.36 per cent and none of the products are removed from the culture, while the latter does not ferment all of the 1 per cent dextrose furnished. It seems possible that the metabolic products other than the H ions are not sufficiently inhibitory to influence greatly the growth until the hydrogen ion concentration approaches the acid limit, but toward the critical acid zone their effect becomes noticeable.

The growth curves as a whole do not agree exactly with the life phases presented by Buchanan ('18). In fact, the diversity of growths produced by varying the hydrogen ion concentration, as illustrated by fig. 11, is so great that one curve can express the growth of *Bacillus coli* in bouillon only when quite definite limitations of conditions are imposed. In a growing culture of an organism like *Bacillus coli* which produces acid from dextrose and alkali in plain bouillon, growth can be con-

trolled to a certain extent by the hydrogen ion concentration, which can in turn be controlled by the amount of dextrose furnished. The initial amount of dextrose determines the amount of acid produced or the maximum hydrogen ion concentration attained. The work of Clark and Lubs, Besson, Ranque and Senez, and the experimental data presented here give a rather definite idea of the action of *Bacillus coli* according to the amount of dextrose in the medium. With .3 per cent or less of dextrose, insufficient acid is produced to kill the organisms; .4 per cent or more is sufficient dextrose to produce acid to P_H 4.9 or better, and the culture becomes sterile in 6 days or less. An amount of dextrose not accurately determined, but between .3 and .4 per cent, probably depending to some extent on the buffer in the medium, should produce just enough acid, between P_H 5.1 and P_H 4.9, depending on the time of exposure, to kill the culture. If insufficient acid to kill the culture is produced, as from .3 per cent or less of dextrose, a reversion of reaction takes place, which Ayers and Rupp have explained with *Bacillus aerogenes* as the formation of alkaline carbonates from the organic acids, especially from the formic and acetic acids. There is a similarity in reaction and in growth curves between *Bacillus aerogenes* and *Bacillus coli*, the main difference appearing to be in the greater acid resistance of *Bacillus aerogenes*. Growth in the cultures where reversion of reaction takes place seems to be typical. One-tenth per cent of dextrose provides a stimulation to growth, but greater amounts produce some evidence of acid inhibition, followed by an increase in growth with the reversion of the reaction and alkaline inhibition between P_H 7.0 and 7.6. The least inhibition is found in a culture in which the hydrogen ion concentration is held in a narrow zone around the neutral point—probably P_H 6.0–7.0 is the best—by adding small amounts of dextrose at frequent intervals. Thus, with *Bacillus coli*, hydrogen ion concentration and growth within limits can be manipulated by the dextrose furnished. The growth curves emphasize not only the value of the initial reaction and composition of the medium, but also the importance in physiological studies of following the changes in hydrogen ion concentration which the growing bacteria produce in their substrates.

Summary

Growth and death of *Bacillus coli* in the culture bouillon of these experiments does not follow a constant curve but is dependent on the hydrogen ion concentration of the medium.

The hydrogen ion concentration of a growing culture of *Bacillus coli* is controlled by the composition of the medium, and particularly by the amount of fermentable carbohydrate present.

The maximum count, determined by the plate method, in the culture with the hydrogen ion concentration controlled is 3,750,000,000 bacteria per cc., as contrasted with a maximum of 281,000,000 bacteria per cc. in the 1 per cent dextrose bouillon with the hydrogen ion concentration uncontrolled.

No investigation was made of the limiting influence of other factors, such as aëration, on the maximum number of bacteria per cc. in the culture where the hydrogen ion concentration was controlled.

No metabolic product of the nature of an "autotoxin" could be found.

Of the products of metabolism, acid is the most inhibitory, checking growth slightly at P_H 5.5 and increasing in intensity to a lethal concentration between P_H 5.1 and 4.9.

The first inhibition on the alkaline side is noted between P_H 7.0 and 7.6, depending on the age of the culture and other factors. P_H 7.6 is comparable in inhibitory action to P_H 5.1. In an asparagin-$CaCO_3$ bouillon, P_H 9.5 is not fatal.

The inhibitory action of the metabolic products of dextrose other than the hydrogen ions is only evident near the critical acid concentration.

To Dr. B. M. Duggar the writer is deeply indebted. His aid and suggestions throughout the investigation have been invaluable.

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THE NUTRITIVE VALUE OF THE FOOD RESERVE IN COTYLEDONS

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Considerable work has been done in respect to determining the capacity for growth of immature and mature seed-plant embryos separated from the endosperm or from the cotyledons. Yet this work seems to have been of comparatively little significance in ascertaining whether, under any conditions of germination and growth, these natural food reserves may be partially or completely substituted for in establishing the seedling with normal vigor in the soil or in the usual culture solutions. Reference will be made later to some of the more important literature bearing on the questions to be presented in this paper. The data here reported are, however, preliminary and intended primarily to give the results of some experiments (1) demonstrating, in those cases where the cotyledons serve as a food reserve, the striking importance of these seed-leaves in comparison with certain organic substances as a source of food for the normal and vigorous establishment of the young plant under cultural conditions, and (2) suggesting the possibility that carbohydrate or hydrocarbon food material stored outside the embryo, as in the case of corn, may be of far less significance.

Doubtless the assumption has been quite generally made that in the case of peas, beans, and other plants in which the cotyledons furnish practically the entire food reserve these seed-leaves may constitute the chief source of organic food until the first green leaves are developed. It has seemed to the writer that interesting physiological problems might be approached through a critical study of the early food reserves, and preliminary tests with Canada field peas confirmed this assumption. Accordingly, the first series of experiments with Canada field peas and with field corn were made merely to determine quantitatively the extent to which the excision of the cotyledons, or of the food supply stored outside of the embryo and the scutellum, influenced normal growth.

In the case of the Canada field peas the seed were germinated on paraffined wire mesh over tap water, and growth was permitted to proceed in diffuse light until the plumules were well established with unfolding green leaves. Solution cultures were then made in the usual way as especially described in an earlier paper (Duggar, '19). All cultures were therefore arranged in duplicate in tumblers holding about 250 cc., and the seedlings were inserted through holes in paraffined paper covers (peas), or through notches in the corks (corn). Seedlings of uniform size were selected and all cultures were placed in the greenhouse, freely and equally exposed to sunlight. A mineral nutrient solution, designated in the paper referred to above as solution B, was employed. It should be observed that this solution contains not merely all essential ions, including NO_3, but contains these in favorable proportions and concentrations for the promotion of excellent growth. The date of the beginning of the experiment was taken as that on which the cultures were exposed in the greenhouse. At intervals of a day or more apart, the cotyledons of successive pairs of cultures were cut away so as to determine their influence on growth, and the time of excision of the last pair represented the practical exhaustion of these food reserves. In the case of corn the young plantlet with attached scutellum was carefully dissected out from the endosperm, an operation which may be effected with very little difficulty after germination begins. In all other respects the corn cultures were treated in precisely the same manner as the peas. The total green weights of all cultures are given in table 1, and the appearance of the peas at the end of the period of observation, 24 days, is shown by pl. 7.

From the results with peas it is clear that for a growth interval of 24 days the removal of the cotyledons after the second day induces a marked depression in the growth rate, and this depression is increasingly less, until, when the removal of the cotyledons occurs after 7 days, the amount of growth is very nearly the same as in the control, with cotyledons intact. Duplication of this experiment with some modifications in the interval led to the conviction that under the conditions the cotyledons are practically exhausted in somewhat less than 10 days. It might be pointed out that the removal of the coty-

DUGGAR—FOOD RESERVE IN COTYLEDONS
(Canada field peas, see table 1)

TABLE I

THE EFFECTS OF THE RESERVE FOOD SUPPLY ON THE GROWTH OF SEEDLINGS

Cult. no.	Field corn, 10 plants, average of duplicate cultures		Cult. no.	Canada field peas, 10 plants, average of duplicate cultures	
	Time of excision of endosperm	Total green wt., grams		Time of excision of cotyledons	Total green wt., grams
1	After 2 days	32.72	1	After 2 days	5.75
2	After 5 days	30.18	2	After 3 days	10.12
3	After 7 days	34.02	3	After 4 days	17.70
4	After 8 days	30.11	4	After 5 days	19.82
5	After 9 days	36.11	5	After 7 days	24.30
6	After 10 days	36.98	6	Control, uncut	25.51

ledons was done in all cases with the greatest care, so that no injury to the seedling would result. The excision was made at a point beyond the stalk of the cotyledons. In the case of corn the results are a little irregular. Nevertheless, there is the suggestion that the removal of the main carbohydrate food supply is not so important a factor in depressing the growth of the young plant. From subsequent incidental experiments I am convinced that there is some effect, but it is neither so marked as in the case of the peas nor does it seem to be so permanent, that is, the effect is not so striking during the further development of the plant.

During the summer of 1919[1] an attempt was made to substitute for the loss of the cotyledons in the case of the peas by the addition of certain organic nitrogen-containing nutrients, and especially by the addition of glycocoll, alanin, sodium asparaginate, and sodium nucleinate. These experiments were carried out under the most favorable conditions for the growth

[1] This series of experiments and other supplementary studies not yet concluded were carried out at the Coastal Laboratory of the Carnegie Institution of Washington, Carmel, California, and the writer takes this occasion to acknowledge his indebtedness to the Director of Botanical Research, Doctor D. T. MacDougal, for placing at his disposal the facilities of the laboratory and for his cordial coöperation.

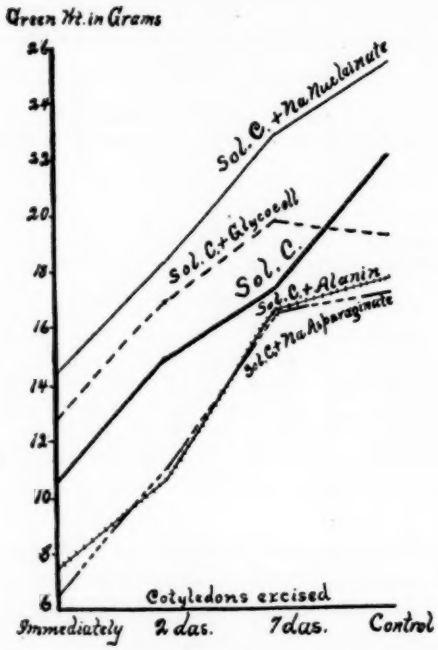

Fig. 1. Green weight quantities of Canada field peas in various solutions as affected by cotyledon excision.

of peas, at a mean temperature of about 15.6° C. The technique was the same as above described, but the use of organic substances in the solutions made it desirable to renew the solutions every 3 or 4 days in order to reduce or control bacterial action. The complete results are shown in table II, and in fig. 1. It will ultimately be necessary to extend the use of organic substances and to repeat this work under pure culture conditions. In fact, a small series of experiments in this direction has already been performed, but inasmuch as these are to constitute a part of a more extended study I will confine myself here to a brief discussion of the data presented below.

In general it will be seen that whatever the medium employed as a nutrient solution the removal of the cotyledons is shown by marked depression in the growth rate. The mineral nutrient solution employed in this case is that which I have in a previous

TABLE II

THE EFFECTS OF THE EXCISION OF THE COTYLEDONS UPON THE GROWTH OF CANADA FIELD PEAS

Cult. no.	Culture medium	Time of excision	Total gr. wt.	Gr. wt. of tops	Gr. wt. of roots	Per cent rel. to cor. cult., sol. C*	Per cent rel. to cult. 4
			10 plants, weight in grams				
1	Solution C	Immediately	10.26	4.97	5.29	100	46
2		After 2 days	14.90	8.75	6.15	100	67
3		After 7 days	17.50	11.64	5.86	100	79
4		Control, uncut	22.11	14.40	7.71	100	100
5	Solution C	Immediately	12.28	7.38	4.90	120	56
6	+	After 2 days	16.90	10.40	6.50	113	76
7	glycocoll	After 7 days	19.81	13.93	5.88	113	90
8	(M/100)	Control, uncut	19.29	13.45	5.84	87	87
9	Solution C	Immediately	6.57	3.92	2.65	64	30
10	+ sodium	After 2 days	11.04	6.68	4.36	74	50
11	asparaginate	After 7 days	16.44	10.09	6.35	94	74
12	(M/100)	Control, uncut	17.26	11.52	5.74	78	78
13	Solution C	Immediately	7.53	4.62	2.91	73	34
14	+	After 2 days	10.69	6.92	3.77	72	48
15	alanin	After 7 days	16.56	10.45	6.11	95	75
16	(M/100)	Control, uncut	17.86	12.00	5.86	81	81
17	Solution C	Immediately	14.38	7.29	7.09	140	65
18	+ sodium	After 2 days	18.32	10.13	8.19	123	83
19	nucleinate	After 7 days	22.85	14.57	8.28	131	103
20	(1/10 per cent)	Control, uncut	25.43	15.09	10.34	115	115

* Per cent relative to corresponding culture, with respect to the time of excision of cotyledons, in the unmodified solution C.

paper (Duggar, '20) called solution C, which is one of the "best" combinations developed by Livingston and Tottingham ('18), and this solution has been shown to contain a favorable concentration of potassium nitrate for the growth of wheat and peas. Nevertheless, in spite of the fact that at the time of the excision of the cotyledons (even in those excised after only 2 days) there was considerable green leaf tissue in the seedlings, still the growth was weak, and at the end of 24 days the plants weighed less than one-half the control. The addition of glycocoll and of sodium nucleinate increased the growth quantities materially in the corresponding cultures, while the addition of sodium asparaginate or alanin was slightly depressing. The depressing

action, however, may have been due to a small amount of bacterial decomposition products. Even in the presence of sodium nucleinate, as in culture 17, the amount of growth when the cotyledons were excised after 2 days is much less than in the control (cotyledons uncut) in solution C, culture 4. The data seem to indicate that no proper nutrient substitute for the cotyledons has been found in these organic substances. Some additional experiments in which sugar was used in connection with nitrogen-containing substances have not served to change materially the conclusions which may be drawn. In other experiments urea and nucleic acid were used, but neither of these has been as favorable as sodium nucleinate or glycocoll. It is true, however, that sodium nucleinate has increased more than any other compound thus far used the growth quantities in the cultures lacking cotyledons.

The importance of the cotyledons in the vigorous development of the seedling is an ancient observation. Bonnet (1754) demonstrated that beans and buckwheat grew less rapidly when the cotyledons were cut off, and more important still, he observed the persisting effect of this early difference, stating the matter in the following words: "La meme différence, ou une différence analogue, a subsisté entre ces Plantes pendant toute la durée de l'accroissement. Il a toujours été facile de distinguer les unes des autres."

Sachs ('59) observed the same fact a century later while devoting more attention to the physiology of absorption and nutrition. Discussing numerous experiments designed to determine the interdependence of organs and tissues in the embryo, Van Tieghem ('73) refers incidentally to the problem here discussed.

While Schmid ('94), Hannig ('04), and Smith ('07) have contributed many interesting observations regarding the nutrition of the embryo and the capacity of different parts to develop or regenerate, these facts do not closely relate to the present investigation. Dubard and Urbain ('13), however, emphasize the favorable effects of the endosperm of certain grains in the early stages of germination. They directed their work primarily toward determining the capacity of the embryos to develop in the absence of the endosperm.

Recently Andronescu ('19) has sought to determine the importance of the endosperm and scutella in *Zea Mays* and at the same time he has endeavored to find a substitute for these, also to follow in heredity any effects observed. While demonstrating that normal plants develop without endosperm, his use of the term normal is a relative one, and he concludes with the statement, "We cannot deny, however, that the presence of endosperms is beneficial in the process of germination, as well as in the further development of the plants."

The writer proposes to continue these investigations with plants grown under sterile conditions in the hope of determining more definitely the nature of the special nutrient or growth-inducing substance furnished by the cotyledons. At present one of several explanations for the failure to substitute readily for the cotyledons may be given: (1) it is conceivable that a combination of organic nutrients including several amino acids may be necessary for normal growth; (2) that penetration of organic substances may be slow and difficult; and (3) that the cotyledons may contain a vitamine requisite for the vigorous development of the plant. In any case the results so far obtained, as well as the observations of other investigators, indicate that the depression of growth accompanying the excision of the cotyledons is marked in the case of peas and other plants with fleshy seed-leaves, and that the influence of excision extends throughout the growth period of the plants.[1]

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[1] Since this paper was written there has appeared another article of considerable interest dealing with the effect of the endosperm upon the growth of the embryo (Urbain, A., Influence des matiéres de réserve de l'albumen de la graine sur le développement de l'embryon. Rev. Gén. Bot. **32**: 125–139, 165–191. *24 fig.* 1920.). In addition to careful observations on the effects of the excision of the endosperm on the development of a number of plants, a careful comparative study was made of internal structure.

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TITRATION CURVES OF CERTAIN LIQUID CULTURE MEDIA

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A study of the growth of various fungi and of the germination of various fungous spores with reference to H-ion concentration has involved a determination of the titration curves of several culture media. It is the purpose of this paper to present briefly the data obtained with these nutrient solutions, which have been the basis of subsequent work in this laboratory.

The formulae for the media employed are as follows:

Beet decoction.—Prepared according to the method outlined by Duggar, Severy, and Schmitz ('17). This consists essentially of 370.4 gms. of sugar beets per liter of distilled water, autoclaved at 15 pounds for one hour, and then filtered.

Czapek's solution.—$MgSO_4+7\ H_2O$, .5 gm.; KH_2PO_4, 1.0 gm.; KCl, .5 gm.; $NaNO_3$, 2.0 gms.; $FeSO_4$, .01 gm.; cane sugar, 30.0 gms.; distilled H_2O, 1000 cc. (Zeller, Schmitz, and Duggar, '19).

Peptone solution.—Twenty gms. bacto-peptone in 1000 cc. H_2O.

Pfeffer's solution.—KH_2PO_4, 5.0 gms.; $MgSO_4+7\ H_2O$, 2.5 gms.; NH_4NO_3, 10.0 gms.; $FeSO_4$, trace; cane sugar, 50.0 gms.; distilled H_2O, 1000 cc. (Pfeffer, '95).

Richards' solution.—KH_2PO_4, .5 gm.; KNO_3, 4.0 gms.; $MgSO_4+7\ H_2O$, .5 gm.; NH_4NO_3, 10.0 gms.; $FeSO_4$, trace; cane sugar, 30.0 gms.; distilled H_2O, 1000 cc. (Richards, '97). This formula differs from that of the original by a reduction in the amount of $MgSO_4$. The amount of precipitate produced in the more alkaline solutions was found to depend largely upon the amount of magnesium present, and, since it is important to keep the amounts of the constituents in the solutions as nearly equal as possible while varying the H-ion concentration, it seemed desirable to reduce the amount of $MgSO_4$ to .5 gm. per liter. Such

a reduction has been found by Duggar (unpublished data) to have no appreciable effect upon the growth of fungi.

The nutrient solutions were all prepared by adding 600 cc., instead of 1000 cc., of distilled water. This method allowed dilutions of the various solutions by additions of regulated amounts of acid or alkali and water for the adjustment of various H-ion concentrations without affecting materially the concentrations of the nutrient salts or constituents.

It is possible to obtain a wide range in the H-ion concentrations of the solutions by adding an alkali and a mineral acid in successively increasing quantities. The addition of the mono-, the di-, or the tri-basic potassium phosphate as suggested by Zeller, Schmitz, and Duggar ('19) was found to give results within a certain range, but additions in too large amounts were necessary to produce extreme concentrations. Therefore, since such a decided variation in the composition of the nutrient solutions undoubtedly would have existed, it seemed undesirable to adopt this method in the experiments under investigation.

From preliminary experimentation relative to the adjustment of H-ion concentration in media of alkaline reactions, the most satisfactory results were obtained by using N/5 KOH, and an alkali of this strength has been employed in all of the experiments except in the case of Czapek's solution. Here, the buffer action was less than that in the other nutrient solutions, consequently N/20 concentration was more conveniently and accurately applicable. In all the experiments, however, N/5 HCl was favorable for varying the reactions on the acid side. In the case of Czapek's solution, the reactions were also varied by means of N/1 H_3PO_4 and N/20 NaOH. These results so nearly paralleled those obtained with the above acid and alkali that it was deemed unnecessary to continue this aspect of the experiment with the other nutrient solutions.

Inasmuch as it is generally admitted that sugars readily react with acid or alkali when heated under pressure, the nutrient solutions and the acid and the alkali were sterilized separately. Thirty cc. of the nutrient solution together with the desired amount of water, as indicated in the tables, were put into small flasks, plugged, and sterilized. After cooling, the

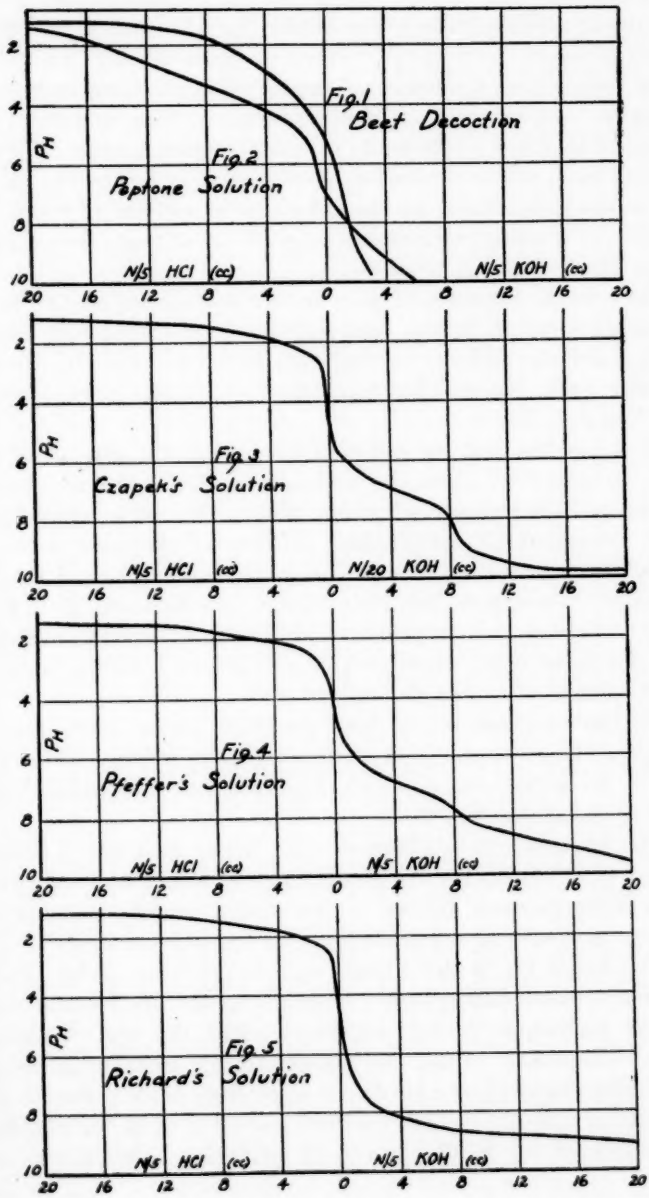
Fig. 1
Beet Decoction
Fig. 2
Peptone Solution
Fig. 3
Czapek's Solution
Fig. 4
Pfeffer's Solution
Fig. 5
Richard's Solution
PH
N/5 HCl (cc)
N/5 KOH (cc)
N/20 KOH (cc)
2
4
6
8
10
20 16 12 8 4 0 4 8 12 16 20

cultures were removed to a culture chamber, and definite amounts of sterile acid and alkali were added by means of sterile graduated pipettes. The final volume of each culture was 50 cc. and represented a dilution of the constituents comparable with that in the original nutrient solutions. The solutions were allowed to stand for 24 hours in order to reach a state of equilibrium, and, at the expiration of this period, H-ion determinations were made according to the colorimetric method of Clark and Lubs ('17), all determinations being made at room temperature. (See figs. 1–5 and tables I–II).

Due to the presence of color in the beet decoction and in the peptone solution, it was necessary to use a colorimeter for the H-ion determinations. A Duboscq type was used in this particular work, the detailed method of which has been described by Duggar ('19).

In all of the mineral salt solutions, a certain amount of precipitate occurred upon the addition of alkali, the amount increasing with increase of added alkali. No such phenomenon was evidenced in the alkaline cultures of the beet decoction or of the peptone solution. The precipitation referred to commenced with culture No. 29 in Pfeffer's solution, No. 27 in Richards' solution, and very faintly in No. 23 of Czapek's solution. On the other hand, there was no precipitation whatever in the acid cultures of any of the various media.

As the reaction of the beet decoction passed from acid to alkaline, there was noted a decided color change from pale yellow to amber, and a slight cloudiness was perceptible beginning with culture No. 42.

The titration curve obtained with 2 per cent bacto-peptone agrees closely with the curves obtained by Clark and Lubs ('17) with Witte peptone, falling, as one would expect, between the curves representing concentrations of 1 and 5 per cent.

The initial P_H of the original culture solutions varied, variations of several tenths not being infrequent despite the most careful technique during preparation and the use of highest purity chemicals. Since the highest purity mono-basic potassium phosphate obtainable had a very high acidity due to the impurities present, the salt was recrystallized until the Sörensen coefficient of P_H 4.529 for a 1/15 molecular solution was obtained.

TABLE I

TITRATION DATA FOR VARIOUS LIQUID MEDIA

No.	Solution (cc.)	N/5 HCl (cc.)	N/5 KOH (cc.)	Dist. H_2O (cc.)	Total (cc.)	Hydrogen ion concentration, P_H			
						Beet decoction	Richards	Peptone	Pfeffer
1	30	20.00		00.00	50	1.2	1.2	1.4	1.4
2	30	15.00		05.00	50	1.2	1.2	2.0	1.5
3	30	10.00		10.00	50	1.6	1.4	3.0	1.5
4	30	9.50		10.50	50		1.4		1.6
5	30	9.00		11.00	50	1.7	1.5	3.2	1.7
6	30	8.50		11.50	50		1.5		1.7
7	30	8.00		12.00	50	1.9	1.5	3.4	1.8
8	30	7.50		12.50	50		1.6		1.8
9	30	7.00		13.00	50	2.1	1.6	3.6	1.9
10	30	6.50		13.50	50		1.6		1.9
11	30	6.00		14.00	50	2.3	1.7	3.8	1.9
12	30	5.50		14.50	50		1.7		2.0
13	30	5.00		15.00	50	2.6	1.7	4.0	2.1
14	30	4.50		15.50	50		1.8		2.1
15	30	4.00		16.00	50	3.1	1.8	4.2	2.1
16	30	3.50		16.50	50		2.0		2.3
17	30	3.00		17.00	50	3.4	2.0	4.4	2.3
18	30	2.50		17.50	50	3.6			2.3
19	30	2.00		18.00	50	3.8	2.2	4.9	2.4
20	30	1.50		18.50	50				2.7
21	30	1.00		19.00	50	4.4	2.4	5.4	2.8
22	30	.75		19.25	50	4.5			
23	30	.50		19.50	50	4.8	2.8	6.4	3.2
24	30	.25		19.75	50	5.0			
25	30	0.00		20.00	50	5.2	4.6	7.0	4.3
26	30		.25	19.75	50	5.4			
27	30		.50	19.50	50	5.6		7.4	5.3
28	30		.75	19.25	50	6.4			
29	30		1.00	19.00	50	7.0	7.0	7.8	5.6
30	30		1.50	18.50	50		7.5		6.2
31	30		2.00	18.00	50	8.8	7.7	8.2	6.3
32	30		2.50	17.50	50		7.9		6.4
33	30		3.00	17.00	50	9.8	8.1	8.8	6.6
34	30		3.50	16.50	50		8.2		6.6
35	30		4.00	16.00	50	10.0+	8.2	9.2	6.8
36	30		4.50	15.50	50		8.3		6.9
37	30		5.00	15.00	50		8.3	9.6	7.0
38	30		5.50	14.50	50		8.4		7.1
39	30		6.00	14.00	50		8.5	10.0	7.2
40	30		6.50	13.50	50		8.6		7.3
41	30		7.00	13.00	50		8.6	10.0	7.4
42	30		7.50	12.50	50		8.7		7.6
43	30		8.00	12.00	50		8.7	10.0+	7.8
44	30		8.50	11.50	50		8.7		8.0
45	30		9.00	11.00	50		8.7	10.0+	8.2
46	30		9.50	10.50	50		8.7		8.3
47	30		10.00	10.00	50		8.8	10.0+	8.4
48	30		15.00	5.00	50		8.9		9.0
49	30		20.00	0.00	50		9.1		9.6

TABLE II

TITRATION DATA FOR CZAPEK'S SOLUTION

No.	Czapek sol. (cc.)	Acid (cc.)	Alkali (cc.)	Dist. H_2O (cc.)	Total cc.	H-ion concentration, P_H	
						N/5 HCl N/20 KOH	N/1 H_3PO_4 N/20 NaOH
1	30	20.00		0.00	50	1.2	
2	30	15.00		5.00	50	1.3	
3	30	10.00		10.00	50	1.4	1.6
4	30	9.50		10.50	50	1.4	1.6
5	30	9.00		11.00	50	1.5	1.6
6	30	8.50		11.50	50	1.5	1.7
7	30	8.00		12.00	50	1.5	1.7
8	30	7.50		12.50	50	1.6	1.7
9	30	7.00		13.00	50	1.6	1.7
10	30	6.50		13.50	50	1.6	1.8
11	30	6.00		14.00	50	1.7	1.8
12	30	5.50		14.50	50	1.7	1.8
13	30	5.00		15.00	50	1.8	1.9
14	30	4.50		15.50	50	1.9	1.9
15	30	4.00		16.00	50	1.9	2.0
16	30	3.50		16.50	50	2.0	2.0
17	30	3.00		17.00	50	2.1	2.1
18	30	2.50		17.50	50	2.2	2.2
19	30	2.00		18.00	50	2.3	2.3
20	30	1.50		18.50	50	2.4	2.3
21	30	1.00		19.00	50	2.5	2.4
22	30	.50		19.50	50	2.9	2.6
23	30	0.00		20.00	50	4.7	4.4
24	30		.50	19.50	50	5.7	5.6
25	30		1.00	19.00	50	6.0	5.9
26	30		1.50	18.50	50	6.2	6.2
27	30		2.00	18.00	50	6.4	6.3
28	30		2.50	17.50	50	6.6	6.5
29	30		3.00	17.00	50	6.7	6.6
30	30		3.50	16.50	50	6.8	6.8
31	30		4.00	16.00	50	6.9	6.8
32	30		4.50	15.50	50	7.0	7.0
33	30		5.00	15.00	50	7.1	7.1
34	30		5.50	14.50	50	7.2	7.2
35	30		6.00	14.00	50	7.4	7.4
36	30		6.50	13.50	50	7.5	7.6
37	30		7.00	13.00	50	7.6	7.7
38	30		7.50	12.50	50	7.7	7.9
39	30		8.00	12.00	50	8.0	8.2
40	30		8.50	11.50	50	8.6	8.6
41	30		9.00	11.00	50	8.9	8.7
42	30		9.50	10.50	50	9.1	9.0
43	30		10.00	10.00	50	9.2	9.2
44	30		15.00	5.00	50	9.8	
45	30		20.00	0.00	50	9.8	

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THE USE OF "INSOLUBLE" SALTS IN BALANCED SOLUTIONS FOR SEED PLANTS

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In this paper it is proposed to give the results of several series of experiments designed primarily to determine the possible value of certain relatively insoluble salts in furnishing the necessary ions for the growth of seed plants. By means of such salts it will also be attempted to secure favorable combinations of the necessary ions. Throughout this discussion "insoluble" may be used in a very general sense, to include many salts soluble only to a comparatively slight degree, or with difficulty soluble, in water at from 15 to 25° C.

It is well known that in the soil a relatively small part of the salts ordinarily designated mineral nutrients is present in soluble form. There is, in general, a very considerable reserve or "unavailable" supply of the less readily soluble salts of such elements as K, Ca, Mg, Fe, S, and P. Nitrates are generally present only in low concentration and the surplus nitrogen supply is usually in the form of organic compounds. It has seemed to the writer eminently desirable to determine, therefore, if a favorable nutrient solution for seed plants may not be arranged from combinations of some of these insoluble salts, thus in some measure imitating the chemical relations in the soil.

In favor of this endeavor it might be argued that should this prove possible it would only be necessary to add to the culture vessel a surplus of the substances required. A small amount of that added would go into solution immediately and when an equilibrium were attained the absorption of any ions by the root would be compensated for by further solution of the substances furnishing these ions, and thus the equilibrium might be maintained and the concentration kept fairly constant over considerable intervals. Obviously, it would be impracticable to furnish nitrate as an insoluble compound, since the nitrates of

the bases required are all soluble to a high degree. If, therefore, nitrogen is furnished as NO_3, the salt furnishing this ion would necessarily be added periodically, and to this extent the concentration would change from day to day. Relatively insoluble salts of ammonium are obtainable, however, such as $MgNH_4PO_4$, and this salt has been employed in some of the experiments.

In the various experiments which have thus far been undertaken the sources of Ca are as follows: $CaSO_4+2H_2O$, $CaCO_3$, $Ca_3(PO_4)_2$, and $CaHPO_4+2H_2O$; of Mg: $MgSO_4+7H_2O$, $MgCO_3$, $Mg_3(PO_4)_2+8H_2O$, and $MgNH_4PO_4+6H_2O$; of K: KNO_3 and K_2PO_4; of Fe: $FePO_4+4H_2O$, $FeC_2O_4+2H_2O$, ferric citrate, and "soluble ferric phosphate." Certain other salts which might have been employed to advantage were not available at the time.

Among the more soluble of the salts included in this category are $CaSO_4+2H_2O$ having a solubility of 0.241 and 0.222 in 100 parts of water at 0° and at 100° C. respectively, and among the more insoluble salts are $FePO_4+4H_2O$ and $CaCO_3$. One of the chief reasons for burdening the experiments with such a variety of substances may be found in the fact of their diverse solubilities; and inasmuch as the antagonistic relations of the ions in respect to the plant require consideration and are involved with osmotic and nutritive relations, such a variety of combinations was necessary in order to feel assured that some of the results obtained might be among the most favorable that such types of combinations could yield.

It is obvious that at no instant is the exact concentration of any salt known in such combinations, except approximately, in cases where soluble salts were added. However, the total concentration is readily determinable, likewise the electrolytic conductivity of the solution. One may also estimate the partial concentrations. When soluble salts are employed in nutrient solutions, the proportion of the ions is disturbed from the moment that the roots are introduced, and there is a progressive decrease in concentration until the solution is renewed. Likewise the differences in the proportion of the ions, determined, of course, by the differential absorption rates, are not continually reestablished by any form of "reserve." It would, of course,

be possible to effect a circulation of fresh nutrient solutions where soluble salts are employed, but any operation of this nature would be impracticable in most of our experimental work. After all, the problem is to obtain a combination of salts favorable to a high degree which may be employed in practically any type of experiment where the desire is to maintain the plant under satisfactory physiological conditions. If the labor involved is great in the one case and small in the other the one involving the less labor will, of course, be selected as the more practicable.

Before describing in detail the methods and experiments to be discussed it should be pointed out that the nutrient solution developed by Crone ('03) and considered by him to have certain advantages over the Pfeffer solution, contained, in addition to KNO_3, $MgSO_4$, and $CaSO_4$, ferrous phosphate and tribasic calcium phosphate. The two salts last mentioned are, of course, relatively insoluble and were used by Crone with the idea of diminishing the chlorosis which he attributed to the excess of soluble phosphate and the low content of iron. Later, however, Benecke ('09) was unable to substantiate the claims made by Crone as to the benefits to be derived from the type of solution which the latter had formulated.

The experiments on which the data in table 1 are based were carried out in the experimental greenhouse at the Missouri Botanical Garden during April, 1920. The methods employed were in fairly close accord with those previously described. Glass tumblers of 250 cc. capacity were used with 240 cc. of nutrient solution. To these containers were fitted corks 7.5 cm. in diameter and 0.7 cm. in thickness, arranged with holes for the insertion of the roots of the seedlings, and with an extra hole to facilitate the addition of water lost by transpiration. The seedlings were germinated over water, and in order to insure the greatest possible uniformity in size a selection of these was made when the shoots were about 2 cm. in height. Each cork was held in position by a stout rubber band passing around the tumbler lengthwise. The tumblers were covered as usual to protect the roots from the light, and the cultures were then freely and equally exposed on a lattice bench in the greenhouse. The variety of wheat employed throughout was the Pacific Coast Blue Stem,

supplied by the Plant Introduction Garden of the Bureau of Plant Industry, Chico, California. The corn was a standard field strain of yellow Dent.

The results shown in tables II and III[1] were obtained at Carmel, California, during July and August of the same year. At Carmel the cultures were arranged on lattice tables in the open. The average temperature was about 15.6° C. and the average daily evaporation from a standard spherical porous cup atmometer about 15 cc. Table II represents cultures prepared exactly as in table I except that glass beakers, of the same capacity, were used instead of tumblers. The data in table III are from experiments closely paralleling those represented by table II except that in the former the containers used were one-quart preserving jars (Economy style). This type of jar proved most convenient in this work, since the mouth of the jar is large, taking the same corks as used in the tumblers and beakers. Moreover, the spring clips which accompany these jars afford a handy method of fastening the cork to the jar so that the seedlings are not readily disturbed. The use of the larger containers in this case explains the larger quantities of salts or solutions employed, and, of course, vessels of this capacity permit the experiments to be continued over a longer period of time.

Inasmuch as certain cultures in each of these series contained not only a full mineral "nutrient" solution but also some citrate, it seemed well to arrange all the solutions and then let them stand two days in case some evidence of fermentation might develop. This occurred in certain cases especially in the second and third series, but afterwards cleared up. The significance of this will be discussed in a later paper in which the physical characteristics of nutrient solutions in general will receive special consideration.

In all cases where readily soluble salts were used in these experiments the initial quantities in the different series varied considerably, as also the quantities added from time to time,

[1] This work was done at the Coastal Laboratory of the Carnegie Institution of Washington. The writer is pleased to make acknowledgment of the facilities and coöperation extended by Doctor D. T. MacDougal, Director of Botanical Research, and of the courtesies of other members of the staff.

and these facts are brought out in the special explanations given in connection with the particular tables.

In table I there are given in the second and third columns (under "concentration," I and II) the quantities of the salts used, these being expressed in grams of the pure salt or in cc. of a standard stock solution. The concentration numbers occur again in the fifth column, indicating to which concentration the data in the remaining columns refer. When given in grams, the quantities indicated were used in 240 cc. of water, and no change of these constituents was made throughout the interval of growth.

The quantities given in cc. also require explanation. KNO_3: the stock solution employed for every culture in which this salt occurs except No. 15 contains 35 grams KNO_3 in 1000 cc. of water, and the use of 10 cc. per culture of 240 cc. gives a concentration of this salt in the culture solution approximately three times as great as in solution B (No. 15 in this table). It is approximately two-thirds as strong as the concentration of KNO_3 in one of the "best" cultures of Livingston and Tottingham ('18), that is, R_4C_1, referred to in my earlier paper (Duggar, '20) as solution C. Moreover, in the two cultures (No. 12 and No. 14) in which $Mg(NO_3)_2$ or $NaNO_3$ was substituted for KNO_3, the strength of the solution was such as to afford a quantity of NO_3 equivalent to that of the KNO_3 in all cultures except No. 15. $MgSO_4$: the stock solution, 12 grams in 1000 cc. of water, is the same as that used in solution B (No. 15 of this series). The concentration of soluble ferric phosphate is likewise made the same as in solution B (No. 15 of this table). The control solution in this series is solution B, No. 15 of the table, previously described in detail, as noted above. Additions of 10 cc. of KNO_3 were made to each culture (240 cc.) containing this salt at intervals of 7 days, and at the same time the solution in No. 15 (solution B, control) was renewed.

A glance at table I, and more especially a study of fig. 1 (wheat), indicates that the differences between the two "concentrations" or strengths of solutions are within the probable limits of variation commonly found in duplicate cultures. The average of the two similar control cultures in solution B (No. 15) is exceeded by No. 2. The latter culture differs from the control

TABLE I

GROWTH OF WHEAT AND CORN IN SOLUTIONS OF RELATIVELY INSOLUBLE SALTS. THE GROWTH QUANTITIES REPRESENT, FOR WHEAT, 10 PLANTS, AND FOR CORN, 8 PLANTS; PERIOD OF CULTURE, 21 DAYS

Cult. No.	Concentration I	Concentration II	Salts used		Total gr. wt. gms.	Kind of plant	Gr. wt. tops gms.	Gr. wt. roots gms.	P_H Init.	P_H Fin.
	.125 gm.	.25 gm.	$CaSO_4+2H_2O$	I	10.20	Wheat	5.70	4.50	6.9	7.5
	.125 gm.	.50 gm.	$Mg_3(PO_4)_2+8H_2O$	II	14.00	Wheat	8.60	5.40	6.9	7.5
1	.125 gm.	.25 gm.	$FePO_4+4H_2O$	I	35.90	Corn	24.20	11.70	6.9	8.4
	10 cc.	10 cc.	KNO_3	II	33.60	Corn	25.20	8.40	6.9	7.3
	.125 gm.	.25 gm.	$CaSO_4+2H_2O$	I	17.10	Wheat	10.10	7.00	7.0	8.5
	.125 gm.	.50 gm.	$Mg_3(PO_4)_2+8H_2O$	II	17.80	Wheat	10.50	7.30	7.0	7.5
2	40 cc.	40 cc.	Sol. ferric phosphate	I	35.85	Corn	25.70	10.15	7.0	8.3
	10 cc.	10 cc.	KNO_3	II	35.95	Corn	26.95	9.00	7.0	7.4
	.25 gm.	.50 gm.	$CaCO_3$	I	10.40	Wheat	5.50	4.90	7.2	7.7
	10 cc.	10 cc.	$MgSO_4+7H_2O$	II	8.45	Wheat	5.10	3.35	7.2	7.4
3	.125 gm.	.25 gm.	$FePO_4+4H_2O$	I	35.40	Corn	22.45	12.95	7.2	7.2
	10 cc.	10 cc.	KNO_3	II	35.25	Corn	26.10	9.15	7.2	7.4
	.25 gm.	.50 gm.	$CaCO_3$	I	15.00	Wheat	8.10	6.90	7.3	8.3
	10 cc.	10 cc.	$MgSO_4+7H_2O$	II	12.40	Wheat	7.60	4.80	7.3	7.5
4	40 cc.	40 cc.	Sol. ferric phosphate	I	41.85	Corn	27.25	14.60	7.3	7.3
	10 cc.	10 cc.	KNO_3	II	36.10	Corn	25.15	10.95	7.3	7.4
	.125 gm.	.25 gm.	$CaSO_4+2H_2O$	I	8.80	Wheat	5.50	3.30	8.5	7.3
	.125 gm.	.25 gm.	$MgCO_3$	II	8.80	Wheat	5.00	3.80	8.0	8.6
5	.125 gm.	.25 gm.	$FePO_4+4H_2O$	I	32.50	Corn	23.85	8.65	8.5	8.5
	10 cc.	10 cc.	KNO_3	II	31.85	Corn	23.10	8.75	8.0	8.5

TABLE I—*Continued*

Cult. No.	Concentration I	Concentration II	Salts used		Total gr. wt. gms.	Kind of plant	Gr. wt. tops gms.	Gr. wt. roots gms.	P_H Init.	P_H Fin.
	.125 gm.	.25 gm.	$CaSO_4+2H_2O$	I	12.00	Wheat	7.60	4.40	8.3	8.7
	.125 gm.	.25 gm.	$MgCO_3$	II	9.70	Wheat	5.90	3.80	7.9	8.7
6	40 cc.	40 cc.	Sol. ferric phosphate	I	38.10	Corn	26.30	11.80	8.3	8.4
	10 cc.	10 cc.	KNO_3	II	34.85	Corn	25.90	8.95	7.9	8.8
	.125 gm.	.25 gm.	$CaHPO_4+2H_2O$	I	12.80	Wheat	6.80	6.60	6.4	8.5
	10 cc.	10 cc.	$MgSO_4+7H_2O$	II	11.45	Wheat	7.20	4.25	6.3	8.3
7	.125 gm.	.25 gm.	$FePO_4+4H_2O$	I	34.06	Corn	23.37	10.69	6.4	8.5
	10 cc.	10 cc.	KNO_3	II	41.45	Corn	28.70	12.75	6.3	7.4
	.125 gm.	.25 gm.	$CaHPO_4+2H_2O$	I	15.60	Wheat	9.00	6.60	6.8	8.8
	10 cc.	10 cc.	$MgSO_4+7H_2O$	II	14.10	Wheat	8.90	5.20	6.6	8.3
8	40 cc.	40 cc.	Sol. ferric phosphate	I	38.29	Corn	26.17	12.17	6.8	8.5
	10 cc.	10 cc.	KNO_3	II	41.90	Corn	27.40	14.50	6.6	8.1
	.125 gm.	.25 gm.	$CaHPO_4+2H_2O$	I	8.10	Wheat	4.20	3.90	5.5	7.5
	10 cc.	10 cc.	$MgSO_4+7H_2O$	II	8.00	Wheat	4.05	3.95	5.3	7.2
9	.125 gm.	.25 gm.	$FeC_2O_4+2H_2O$	I	25.55	Corn	17.95	7.60	5.5	8.5
	10 cc.	10 cc.	KNO_3	II	26.25	Corn	18.35	7.90	5.3	8.3
	.25 gm.	.50 gm.	$MgNH_4PO_4+6H_2O$	I	14.00	Wheat	8.90	5.10	7.3	7.4
	.125 gm.	.25 gm.	$CaSO_4+2H_2O$	II	15.90	Wheat	10.90	5.00	7.2	7.2
10	40 cc.	40 cc.	Sol. ferric phosphate	I	34.15	Corn	22.45	11.70	7.3	8.3
	10 cc.	10 cc.	KNO_3	II	35.45	Corn	25.80	9.65	7.2	7.4

TABLE I—*Continued*

Cult. No.	Concentration I	Concentration II	Salts used		Total gr. wt. gms.	Kind of plant	Gr. wt. tops gms.	Gr. wt. roots gms.	P_H Init.	P_H Fin.
11	.25 gm.	.50 gm.	$MgNH_4PO_4+6H_2O$	I	7.00	Wheat	4.70	2.30	7.1	7.4
	.125 gm.	.25 gm.	$CaSO_4+2H_2O$	II	7.10	Wheat	4.90	2.20	7.1	7.4
	.125 gm.	.25 gm.	$FePO_4+4H_2O$	I	31.60	Corn	24.00	7.60	7.1	7.6
	10 cc.	10 cc.	KNO_3	II	34.63	Corn	27.53	7.10	7.1	7.3
12	.25 gm.	.50 gm.	K_3PO_4	I	4.60	Wheat	2.80	1.80	7.2	7.3
	.125 gm.	.25 gm.	$CaSO_4+2H_2O$	II	2.61	Wheat	1.61	1.00	7.4	7.4
	.125 gm.	.50 gm.	$FePO_4+4H_2O$	I	17.65	Corn	13.80	3.85	7.2	8.5
	10 cc.	10 cc.	$Mg(NO_3)_2+6H_2O$	II	16.80	Corn	12.60	4.20	7.4	7.5
13	.25 gm.	.50 gm.	K_3PO_4	I	5.67	Wheat	3.89	1.78	8.6	7.6
	.125 gm.	.25 gm.	$CaSO_4+2H_2O$	II	5.33	Wheat	3.72	1.61	8.6	7.6
	.125 gm.	.25 gm.	$FeC_2O_4+2H_2O$	I	25.55	Corn	19.15	6.40	8.6	8.6
	.25 gm.	.50 gm.	$MgNH_4PO_4+6H_2O$	II	26.70	Corn	20.30	6.40	8.6	7.4
14	.25 gm.	.50 gm.	K_3PO_4	I	2.15	Wheat	1.60	.55	9.0+	8.2
	.125 gm.	.25 gm.	$CaSO_4+2H_2O$	II	——	Wheat	——	——	9.0+	8.3
	.125 gm.	.25 gm.	$FePO_4+4H_2O$	I	14.90	Corn	11.50	2.40	9.0+	8.4
	.25 gm.	.50 gm.	$MgNH_4PO_4+6H_2O$	II	12.90	Corn	8.45	4.45	9.0+	8.4
	10 cc.	10 cc.	$NaNO_3$							
15	30 cc.	30 cc.	$CaSO_4+2H_2O$	I	14.70	Wheat	7.80	6.90	6.9	7.3
	10 cc.	10 cc.	$MgSO_4+7H_2O$	II	17.35	Wheat	9.75	7.60	6.9	7.6
	40 cc.	40 cc.	Sol. ferric phosphate	I	30.60	Corn	20.50	10.10	6.9	7.5
	10 cc.	10 cc.	KNO_3	II	25.40	Corn	17.80	7.60	6.9	7.2

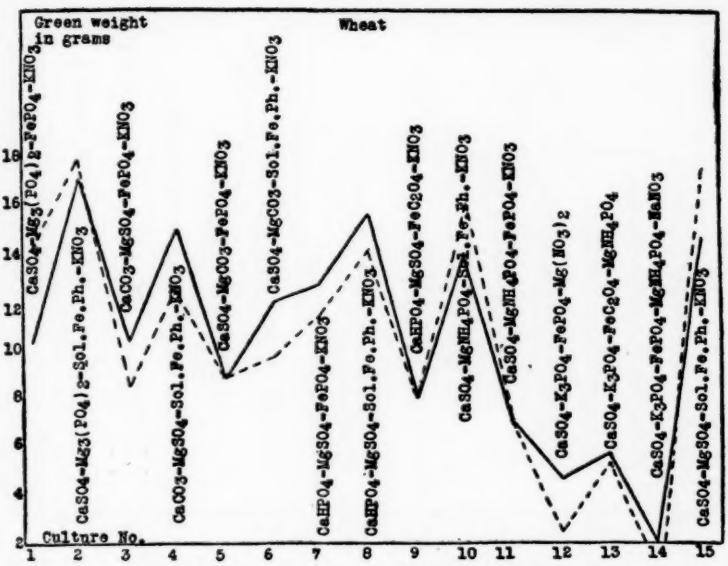

Fig. 1. Yield of wheat in solutions of relatively insoluble salts. Continuous line is concentration I and broken line concentration II (see table I).

(1) in the excess of $CaSO_4$ (solid phase present), (2) in the substitution of the "insoluble" tribasic magnesium phosphate for the soluble $MgSO_4$, and (3) in the greater content of KNO_3. It will be noticed also that cultures 4, 8, and 10 approach the value of the control, and all of these contain the soluble ferric phosphate, combined with various calcium and magnesium compounds. No culture containing either K_2PO_4 or iron oxalate has yielded satisfactorily. Similarly, $MgCO_3$ in the combinations employed would seem to be less depressing than K_2PO_4 but still unfavorable. Among the cultures mentioned as giving the higher yields no striking peculiarity was noted except in the case of No. 10, in which there was pronounced tillering at a relatively early period.

With corn many cultures are ahead of the control, No. 15, and those in advance are again generally the cultures containing soluble ferric phosphate, though the differences between the pairs containing $FePO_4$ and the salt of iron just mentioned

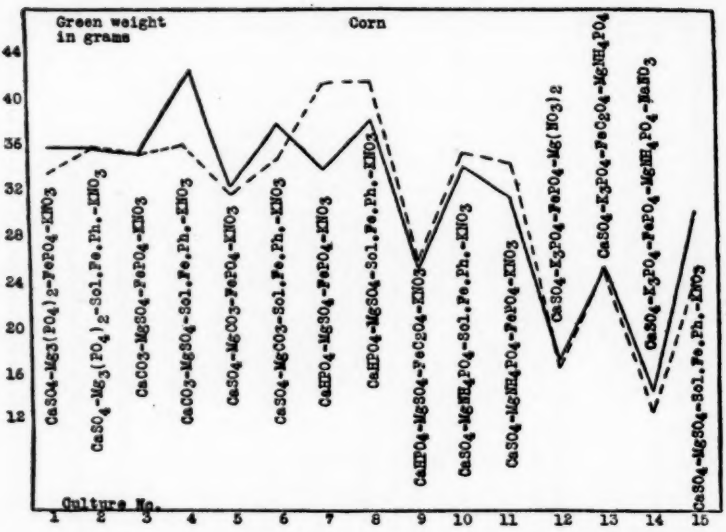

Fig. 2. Yield of corn in solutions of relatively insoluble salts. Continuous line is concentration I and broken line concentration II (see table 1).

are not so striking as with wheat. Nor is it to be assumed that this relation will necessarily hold under all conditions. Moreover, corn, notably resistant to Mg salts, does not exhibit some of the antagonistic effects evident in the case of wheat. No. 11 showed pronounced chlorosis, followed, but to a somewhat less extent, by Nos. 7, 1, 12, 10, 13, and 14. Nos. 2–6, 8, and 15 were normal in appearance, while No. 9 was intensely green.

Difference in "concentration" in the first series was wholly in respect to a variation in the quantity of the relatively insoluble salts; but inasmuch as a considerable amount of the insoluble residue remained in each culture at the close of the experiment it would seem improbable that any difference in the amount of the solid phase would affect the yields. Accordingly, in the series carried out at Carmel, table II and fig. 3, it will be seen that the following are practically the only ways in which the "concentrations" are varied: (1) in column "II" the quantity of KNO_3 is one-half the amount used in column "I," and (2) in column "III," while the amount of nitrate remains

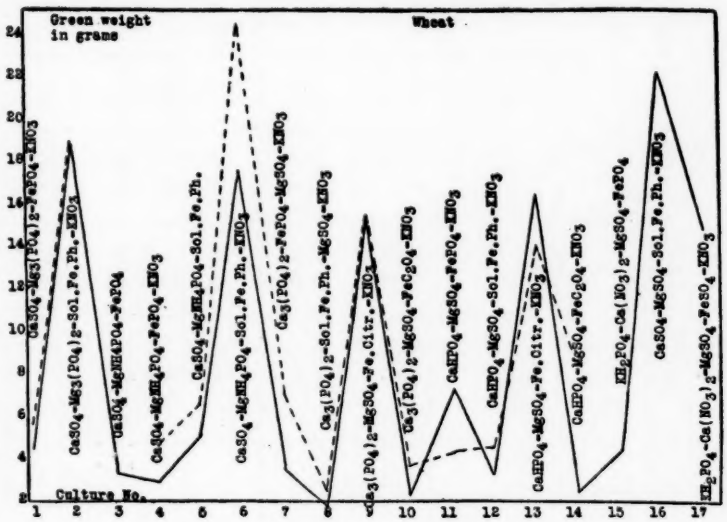

Fig. 3. Yield of wheat in solutions of relatively insoluble salts. Continuous line is concentration I and broken line concentration II (see table II).

as in "I," the quantity of the iron salt is reduced to one-half. The concentration of KNO_3 was the same as in the experiments given in table I. The stock solution of $MgSO_4$ contained 41.932 grams of the salt in 1 liter of water, so that 10 cc. per culture of 240 cc. gave a solution two-thirds as strong in $MgSO_4$ as the R_4C_1 of Livingston and Tottingham ('18). In almost every instance where the growth quantities are small the lower concentrations of the potassium nitrate and of the magnesium sulphate have a tendency within the same culture number to promote the greater growth. In this series the best culture containing insoluble salts is No. 6 ($CaSO_4$ [solid phase present], $MgNH_4PO_4$, soluble ferric phosphate, and KNO_3), followed closely by culture 2, the latter being the same that proved so satisfactory in the previous series.

As will be pointed out later, the more insoluble calcium salts give the higher yields when in combination with relatively insoluble salts of magnesium. This is true except in certain cases where iron citrate enters into the combination. In certain cul-

TABLE II

GROWTH OF WHEAT IN SOLUTIONS OF RELATIVELY INSOLUBLE SALTS. THE GROWTH QUANTITIES REPRESENT 6 PLANTS: PERIOD OF CULTURE, 30 DAYS; CULTURE VOLUME, 240 cc.

Cult. No.	Concentration			Salts used		Total gr. wt. gms.	Gr. wt. tops gms.	Gr. wt. roots gms.	P_H	
	I	II	III						Init.	Fin.
1	.125 gm. .25 gm. .125 gm. 10 cc.	.125 gm. .25 gm. .125 gm. 5 cc.		$CaSO_4+2H_2O$ $Mg_3(PO_4)_2+8H_2O$ $FePO_4+4H_2O$ KNO_3	I II	4.49 5.77	2.62 3.61	1.87 2.16	6.6 —	7.7 7.7
2	.125 gm. .25 gm. .25 gm. 10 cc.	.125 gm. .25 gm. .25 gm. 5 cc.		$CaSO_4+2H_2O$ $Mg_3(PO_4)_2+8H_2O$ Sol. ferric phosphate KNO_3	I II	18.76 18.77	12.76 12.98	6.00 5.79	6.8 —	8.0 8.0
3	.125 gm. .125 gm. .125 gm.			$CaSO_4+2H_2O$ $MgNH_4PO_4+6H_2O$ $FePO_4+4H_2O$	I	3.37	1.35	2.02	7.3	6.5
4	.125 gm. .125 gm. .125 gm. 10 cc.	.125 gm. .125 gm. .125 gm. 5 cc.		$CaSO_4+2H_2O$ $MgNH_4PO_4+6H_2O$ $FePO_4+4H_2O$ KNO_3	I II	2.86 4.77	1.73 2.95	1.13 1.82	7.4 —	7.6 8.0
5	.125 gm. .125 gm. .25 gm.	.125 gm. .125 gm. .125 gm.		$CaSO_4+2H_2O$ $MgNH_4PO_4+6H_2O$ Sol. ferric phosphate	I II	5.11 6.63	2.40 3.02	2.71 3.61	6.3 —	7.3 6.9

TABLE II—*Continued*

Cult. No.	Concentration I	Concentration II	Concentration III	Salts used		Total gr. wt. gms.	Gr. wt. tops gms.	Gr. wt. roots gms.	P_H Init.	P_H Fin.
6	.125 gm.	.125 gm.		$CaSO_4+2H_2O$						
	.125 gm.	.125 gm.		$MgNH_4PO_4+6H_2O$						
	.25 gm.	.25 gm.		Sol. ferric phosphate	I	17.58	12.55	5.03	6.3	8.0
	10 cc.	5 cc.		KNO_3	II	24.50	16.45	8.05	—	8.0
7	.25 gm.	.25 gm.		$Ca_3(PO_4)_2$						
	.125 gm.	.125 gm.		$FePO_4+4H_2O$						
	10 cc.	5 cc.		$MgSO_4+7H_2O$	I	3.56	2.20	1.36	6.4	7.8
	10 cc.	5 cc.		KNO_3	II	7.09	4.49	2.60	6.4	7.9
8	.25 gm.	.25 gm.	.25 gm.	$Ca_3(PO_4)_2$						
	.25 gm.	.25 gm.	.125 gm.	Sol. ferric phosphate	I	1.68	.98	.70	6.2	8.0
	10 cc.	5 cc.	10 cc.	$MgSO_4+7H_2O$	II	2.60	1.75	.85	6.0	8.0
	10 cc.	5 cc.	10 cc.	KNO_3	II	1.68	.99	.69	—	7.8
9	.25 gm.	.25 gm.	.25 gm.	$Ca_3(PO_4)_2$						
	.25 gm.	.25 gm.	.125 gm.	Ferric citrate	I	15.26	9.58	5.68	5.8	8.0
	10 cc.	5 cc.	10 cc.	$MgSO_4+7H_2O$	II	15.60	10.36	5.24	5.7	8.0
	10 cc.	5 cc.	10 cc.	KNO_3	III	8.88	5.25	3.63	—	7.9
10	.25 gm.	.25 gm.	.25 gm.	$Ca_3(PO_4)_2$						
	.125 gm.	.125 gm.	.0625 gm.	$FeC_2O_4+2H_2O$	I	2.27	1.42	.85	5.8	7.7
	10 cc.	5 cc.	10 cc.	$MgSO_4+7H_2O$	II	3.76	2.36	1.40	5.8	7.8
	10 cc.	5 cc.	10 cc.	KNO_3	III	2.01	1.30	.71	—	8.0

TABLE II—*Continued*

Cult. No.	Concentration I	Concentration II	Concentration III	Salts used	Total gr. wt. gms.	Gr. wt. tops gms.	Gr. wt. roots gms.	P_H Init.	P_H Fin.
11	.125 gm. .125 gm. 10 cc. 10 cc.	.125 gm. .125 gm. 5 cc. 5 cc.		$CaHPO_4+2H_2O$ $FePO_4+4H_2O$ $MgSO_4+7H_2O$ KNO_3	I 7.22 II 4.43	4.62 2.97	2.60 1.46	6.4 6.6	8.0 8.0
12	.125 gm. .25 gm. 10 cc. 10 cc.	.125 gm. .25 gm. 5 cc. 5 cc.	.125 gm. .125 gm. 10 cc. 10 cc.	$CaHPO_4+2H_2O$ Sol. ferric phosphate $MgSO_4+7H_2O$ KNO_3	I 3.25 II 4.61 III 6.08	2.18 2.80 3.73	1.07 1.81 2.35	5.6 5.6 —	7.8 7.9 8.0
13	.125 gm. .25 gm. 10 cc. 10 cc.	.125 gm. .25 gm. 5 cc. 5 cc.	.125 gm. .125 gm. 10 cc. 10 cc.	$CaHPO_4+2H_2O$ Ferric citrate $MgSO_4+7H_2O$ KNO_3	I 16.41 II 13.96 III 17.85	11.36 9.60 12.67	5.05 4.36 5.18	5.8 5.8 —	8.0 7.9 8.0
14	.125 gm. .125 gm. 10 cc. 10 cc.	.125 gm. .125 gm. 5 cc. 5 cc.	.125 gm. .0625 gm. 10 cc. 10 cc	$CaHPO_4+2H_2O$ $FeC_2O_4+2H_2O$ $MgSO_4+7H_2O$ KNO_3	I 2.41 II 8.43 III 4.71	1.46 4.77 2.70	.95 3.66 2.01	5.6 5.6 —	7.6 8.0 7.6
15				Solution A	4.56	2.87	1.69	—	5.2
16				Solution B	22.20	13.62	8.58	—	7.6
17				Tottingham's sol.	14.49	9.95	4.54	—	6.4

tures where the magnesium salt is the more soluble, Nos. 8–13, the more favorable action of ferric citrate as contrasted with the soluble ferric phosphate and $FePO_4$ in these cultures with wheat is clearly shown. With the exceptions noted the favorable influence of soluble ferric phosphate in the solution is evident, especially in Nos. 2 and 6, as also, of course, in solution B.

The Tottingham solution was exceeded by 5 combinations. The Shive solution, solution A (No. 15) was unsatisfactory in this series, since after being set up it was found that the acidity was much higher than usual. In these experiments, however, no recrystallization of the salts employed was carried out and no corrections for acidity were made.

The six cultures giving the higher yields (Nos. 2, 6, 9, 13, 16, and 17) were all green and healthy in appearance. Cultures 3 and 5, without KNO_3, were characterized by marked attenuation; No. 14 exhibited excessive greening; and Nos. 7 and 15 were abnormally stocky in general appearance.

As stated previously, the experiments shown in table III and in fig. 4 were also obtained at Carmel. The experiments were set up on July 10, using wheat as a test plant and employing quart jars as containers. For cultures 1–20 the same stock solutions of KNO_3 and $MgSO_4$ as described for table II were used. The results are not in entire agreement with those given in table II. This may be accounted for in part by the use of the larger containers and also in part by differences in weather conditions. During the progress of the experiments here discussed, there were several days of comparatively warm weather without fog, inducing high evaporation rates. It is well to note also that a slight mishap to culture 2, which was found upset one morning, may be responsible in some measure for the low yield of this culture.

Renewals of the solutions in the control cultures (Nos. 21–23) were made about every 10 days. Additional amounts of KNO_3, 20 cc. in the case of all cultures in column "I" and 10 cc. in the case of column "II," were added on July 24 and August 6. No additional $MgSO_4$ was added to the cultures receiving this salt until August 6, when 10 cc. were given each of those receiving this salt in column "I," and 5 cc. for similar cultures in column "II." With the larger amount of nitrate employed, cul-

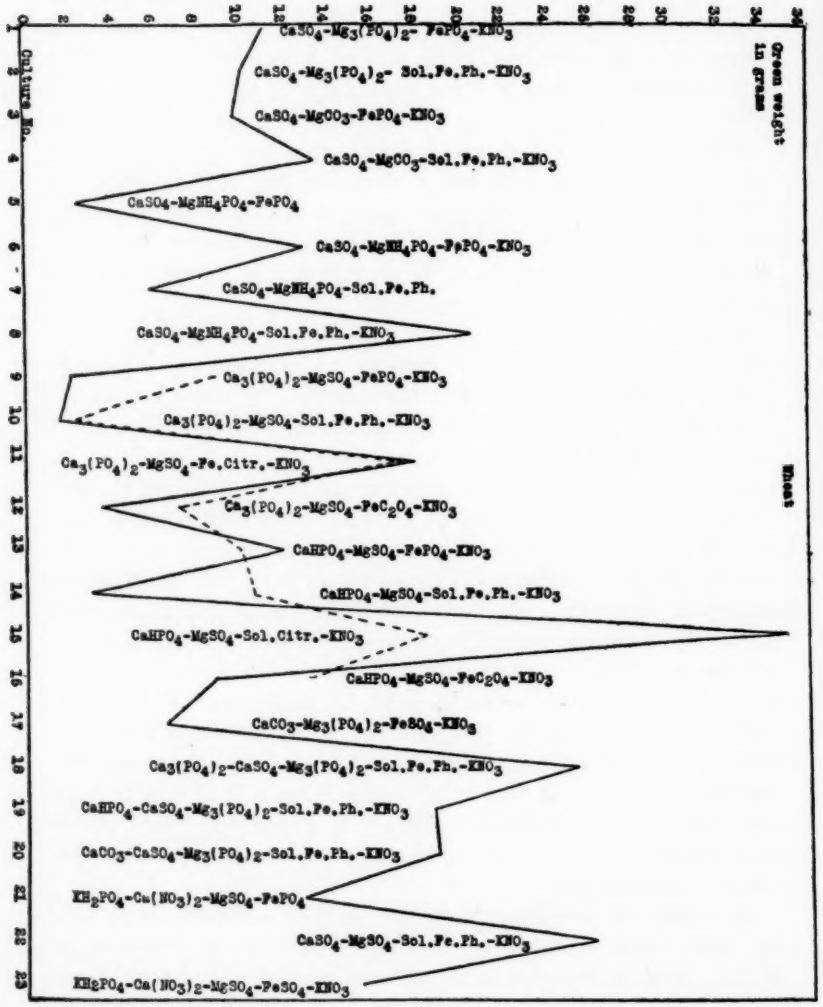

Fig. 4. Yield of wheat in solutions of relatively insoluble salts. Continuous line is concentration I and broken line concentration II (see table III).

ture 15 ($CaHPO_4$, $MgSO_4$, ferric citrate, and KNO_3) is best, followed by one of the controls, solution B, and this in turn is very closely followed by Nos. 18, 8, 20, 19, and 11. Cultures 11 and 15 confirm the previous experience that the ferric citrate effects a high degree of balance in cases where the magnesium salt is more soluble than the calcium salt used; and neither the soluble ferric phosphate nor the $FePO_4$ can replace it in this respect where wheat is the test plant (compare with the cultures above mentioned Nos. 9, 10, 13, and 14).

With due consideration of the causes already mentioned the value of the soluble ferric phosphate in the culture media is confirmed, and the importance of ferric citrate established in certain combinations. No experiments thus far made have thrown any special light on the nature of the benefit derived in these cases from the soluble ferric phosphate or the iron citrate. In both cases, however, within the range of reaction involved in the studies here reported, suspension films consisting in part at least of ferric hydroxide are thrown down. The writer is now endeavoring to determine if floating particles of this type, or a substance in the colloidal state may possibly be of importance in the absorption and distribution of the ions. I have previously mentioned this possibility (Duggar, '20, p. 42) while referring especially to certain experiments of Bonazzi and of Allen on the culture of microörganisms.

Summary

The value of certain relatively insoluble salts as sources of the necessary ions for the growth of seed plants has been tested in a variety of combinations covering by no means, however, the entire range of possibility.

It is argued that in certain types of work many advantages may accrue from the use of combinations of insoluble salts, because of (1) the tendency to maintain a constant concentration of the various ions furnished, and also because (2) no renewal of the solution (except as to the addition of NO_3) is required from day to day.

As sources of Ca, Mg, Fe, PO_4, SO_4, many insoluble salts have been tested, but no salt of this type is procurable as a practical source of NO_3, so that in most experiments this ion is furnished by KNO_3.

TABLE III

GROWTH OF WHEAT IN SOLUTIONS OF RELATIVELY INSOLUBLE SALTS. THE GROWTH QUANTITIES REPRESENT 8 PLANTS: PERIOD OF CULTURE, 40 DAYS; VOLUME OF CULTURE SOLUTION, 890 cc.

Cult. No.	Concentration I	Concentration II	Salts used	Total gr. wt. gms.	Gr. wt. tops gms.	Gr. wt. roots gms.	P_H Init.	P_H Fin.
1	.5 gm. .5 gm. 1.0 gm. 40 cc.		$CaSO_4+2H_2O$ $FePO_4+4H_2O$ $Mg_3(PO_4)_2+8H_2O$ KNO_3	I 11.20	6.58	4.62	7.0	7.8
2	.5 gm. 1.0 gm. 1.0 gm. 40 cc.		$CaSO_4+2H_2O$ Sol. ferric phosphate $Mg_3(PO_4)_2+8H_2O$ KNO_3	I 10.20	6.01	4.19	8.0	7.9
3	.5 gm. .5 gm. .5 gm. 40 cc.		$CaSO_4+2H_2O$ $FePO_4+4H_2O$ $MgCO_3$ KNO_3	I 9.86	5.79	4.07	9.0	7.6
4	.5 gm. 1.0 gm. .5 gm. 40 cc.		$CaSO_4+2H_2O$ Sol. ferric phosphate $MgCO_3$ KNO_3	I 13.47	8.85	4.62	8.8	8.0
5	.5 gm. .5 gm. .5 gm.		$CaSO_4+2H_2O$ $FePO_4+4H_2O$ $MgNH_4PO_4+6H_2O$	I 2.40	1.42	.98	7.1	6.4
6	.5 gm .5 gm .5 gm. 40 cc.		$CaSO_4+2H_2O$ $FePO_4+4H_2O$ $MgNH_4PO_4+6H_2O$ KNO_3	I 12.90	7.72	5.18	6.9	7.4
7	.5 gm. 1.0 gm. .5 gm.		$CaSO_4+2H_2O$ Sol. ferric phosphate $MgNH_4PO_4+6H_2O$	I 5.85	2.62	3.23	7.3	7.3
8	.5 gm. 1.0 gm. .5 gm. 40 cc.		$CaSO_4+2H_2O$ Sol. ferric phosphate $MgNH_4PO_4+6H_2O$ KNO_3	I 20.72	12.05	8.67	8.1	7.2
9	1.0 gm. .5 gm. 40 cc. 40 cc.	1.0 gm. .5 gm. 20 cc. 20 cc.	$Ca_3(PO_4)_2$ $FePO_4+4H_2O$ $MgSO_4+7H_2O$ KNO_3	I 2.08 II 8.83	1.28 5.77	.80 3.06	—	7.6 7.4
10	1.0 gm. 1.0 gm. 40 cc. 40 cc.	1.0 gm. 1.0 gm. 20 cc. 20 cc.	$Ca_3(PO_4)_2$ Sol. ferric phosphate $MgSO_4+7H_2O$ KNO_3	I 1.64 II 2.32	1.11 1.72	.53 .60	8.0	7.7 7.7

TABLE III—*Continued*

Cult. No.	Concentration I	Concentration II	Salts used	Total gr. wt. gms.	Gr. wt. tops gms.	Gr. wt. roots gms.	P_H Init.	P_H Fin.
11	1.0 gm. 1.0 gm. 40 cc. 40 cc.	1.0 gm. 1.0 gm. 20 cc. 20 cc.	$Ca_3(PO_4)_2$ Ferric citrate $MgSO_4+7H_2O$ KNO_3	I 18.02 II 17.31	11.97 9.86	6.05 7.45	8.3	7.9 8.0
12	1.0 gm. .5 gm. 40 cc. 40 cc.	1.0 gm. .5 gm. 20 cc. 20 cc.	$Ca_3(PO_4)_2$ $FeC_2O_4+2H_2O$ $MgSO_4+7H_2O$ KNO_3	I 3.48 II 7.12	2.12 3.67	1.36 3.45	6.2	7.5 7.6
13	.5 gm. .5 gm. 40 cc. 40 cc.	.5 gm. .5 gm. 20 cc. 20 cc.	$CaHPO_4+2H_2O$ $FePO_4+4H_2O$ $MgSO_4+7H_2O$ KNO_3	I 11.87 II 10.00	7.72 6.83	4.15 3.17	6.6	7.6 7.5
14	.5 gm. 1.0 gm. 40 cc. 40 cc.	.5 gm. 1.0 gm. 20 cc. 20 cc.	$CaHPO_4+2H_2O$ Sol. ferric phosphate $MgSO_4+7H_2O$ KNO_3	I 2.97 II 10.67	2.07 7.19	.90 3.48	7.9	7.9 7.8
15	.5 gm. 1.0 gm. 40 cc. 40 cc.	.5 gm. 1.0 gm. 20 cc. 20 cc.	$CaHPO_4+2H_2O$ Ferric citrate $MgSO_4+7H_2O$ KNO_3	I 35.30 II 18.55	22.63 11.13	12.67 7.42	8.1 7.3	8.0 7.9
16	.5 gm. .5 gm. 40 cc. 40 cc.	.5 gm. .5 gm. 20 cc. 20 cc.	$CaHPO_4+2H_2O$ $FeC_2O_4+2H_2O$ $MgSO_4+7H_2O$ KNO_3	I 8.80 II 13.14	5.99 3.09	2.81 6.00	6.7	7.9 7.8
17	1.0 gm. trace .5 gm. 40 cc.		$CaCO_3$ $FeSO_4$ $Mg_3(PO_4)_2+8H_2O$ KNO_3	I 6.52	4.20	2.32	7.8	7.9
18	1.0 gm. .5 gm. 1.0 gm. .5 gm. 40 cc.	1.0 gm. .5 gm. 1.0 gm. .5 gm. 20 cc.	$Ca_3(PO_4)_2$ $CaSO_4+2H_2O$ Sol. ferric phosphate $Mg_3(PO_4)_2+8H_2O$ KNO_3	I 25.55	16.82	8.73	8.0	7.9
19	.5 gm. .5 gm. 1.0 gm. .5 gm. 40 cc.		$CaHPO_4+2H_2O$ $CaSO_4+2H_2O$ Sol. ferric phosphate $Mg_3(PO_4)_2+8H_2O$ KNO_3	I 18.99	12.58	6.41	7.8	8.0

TABLE III—*Continued*

Cult. No.	Concentration I	Concentration II	Salts used	Total gr. wt. gms.	Gr. wt. tops gms.	Gr. wt. roots gms.	P_H Init.	P_H Fin.
20	1.0 gm. .5 gm. 1.0 gm. .5 gm. 40 cc.		$CaCO_3$ $CaSO_4+2H_2O$ Sol. ferric phosphate $Mg_3(PO_4)_2+8H_2O$ KNO_3	1 19.15	12.50	6.65	8.0	7.9
21			Solution A	12.90	7.92	4.98	4.1	5.7
22			Solution B	26.23	15.16	11.07	6.6	7.7
23			Tottingham's sol.	15.55	9.20	6.35	5.8	6.4

A relatively insoluble source of NH_4 ($MgNH_4PO_4$) has been found unsatisfactory as a source of nitrogen with the test plants used.

In each of three series of cultures in which wheat or wheat and corn were used, one or more of the combinations containing two or more insoluble salts exceeded the growth in the best control culture employed. The best control culture contained $CaSO_4$, $MgSO_4$, soluble ferric phosphate, and KNO_3. Cultures exceeding the control contained in the several series the following combinations of salts: I, $CaSO_4$ (solid phase present), $Mg_3(PO_4)_2$, soluble ferric phosphate, and KNO_3; II, $CaSO_4$ (solid phase present), $MgNH_4PO_4$, soluble ferric phosphate, and KNO_3; III, $CaHPO_4$, $MgSO_4$, ferric citrate, and KNO_3.

In all series, with the test plants mentioned, a group of cultures approached very closely the yields of the best combinations, and in all cases in such best combinations the calcium salt is relatively more soluble than the magnesium salt, except in certain combinations into which ferric citrate enters.

Soluble ferric phosphate has proved a valuable constituent in the culture medium in a variety of combinations. In certain

cases ferric citrate has proved equally valuable. Certain fermentation processes may occur in cultures in which these compounds are employed and a further study of the influence of those changes is necessary.

Except in the cultures containing K_2PO_4 or $MgCO_3$, the hydrogen-ion concentration of all combinations used in the three series here reported ranges from 5.6 to 8.0, and after the growth of test plants there is usually a shift in the P_H toward alkalinity or greater alkalinity.

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GENERAL INDEX TO VOLUME VII

New scientific names of plants and the final members of new combinations are printed in **bold face** type; synonyms and page numbers having reference to figures and plates, in *italic;* and previously published scientific names and all other matter, in ordinary type.

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